

Farewell (not fond) to cold fusion

A year after the famous Utah press conference, cold fusion is a diminishing focus for professional belief. The authors of last year's fuss now have a responsibility to make the details of their work public.

If the long history of scientific endeavour is ever written, the past year's fuss about cold fusion will deserve a waspish footnote. Here, without copyright charge, is a model with which historians may conjure:

On 23 March, 1989, two chemists — one British, one American — told a press conference at the University of Utah that they had demonstrated the reality of thermonuclear fusion in a simple electrochemical cell. Even the journalists present would not have believed them were it not for their claims to have detected tell-tale neutrons and γ -rays, which claims were quickly withdrawn or shown to be insubstantial. Even so, the search for what was called "cold fusion" continued for several years, much as the search for the Philosophers' Stone (*q.v.*) persisted in the face of repeated failure and enlarging common-sense, in this case sustained by cash from the State of Utah and grant-making agencies that should have known better. The incident is remembered as that which most directly gave the lie to the doctrine, often then referred to as a cornerstone of late twentieth century science, that the first duty of researchers claiming new discoveries is to make the details available for the scrutiny of others.

The last sentence may have to be modified if Pons and Fleischmann do indeed make a clean breast of their work at the conference that will now have begun at Salt Lake City, and which closes on Saturday this week.

Even so, as the article on page 375 makes plain, the cold fusion fuss is discreditable to the scientific community as a whole. The reasons are plain. First, it has licensed magic in the particular sense that reports of remarkable phenomena — it could next be unicorns again — claim equal credence even when they fly in the face of expectation. Second, by extension, it has shown up the frailty of the collective confidence in theoretical science; why else should so many serious people have been bamboozled for so long? Third, it has revealed the malign influence of extraneous considerations in modern science; Pons and Fleischmann would surely have published a full account of their work long before this if they had been concerned with the general understanding. Nobody will blame them for having hugged what they considered a great discovery to themselves (although that is the chief reason why they were themselves misled) or for having held a press conference, but it is mystifying that they have met scepticism mostly with silence. Finally, it is a shabby example for the young; who can now hope to go about the world telling the tale that science is a collective enterprise in which all shoulders are bent to the same wheel of winning understanding from a common literature without fearing the shout "What about cold fusion?"

In all the circumstances, it is remarkable that the first year has passed off more or less peacefully, and with good humour. Even those diverted from previous pursuits by attempts at replication have mostly been philosophical about the time wasted. Palladium saturated with its atomic equivalent of hydrogen or deuterium is, after all, an interesting material, electrochemistry is an important field in which too few people are engaged, while the intricacies of nuclear measurements at the limits at which particles can be detected have been, for many people, absorbing. If there had been more information to go on, the chase might have been less interesting, if no more rewarding. But there is a limit to people's patience, which has probably been reached with the organization of the first "annual" cold fusion conference.

In short, the time has come when Pons and Fleischmann should say openly, and in as much detail as their interlocutors in Utah this week require, exactly what they had done a year ago, what they have been doing since and what reason they have in which others can have confidence for believing that cold fusion is still to be taken seriously. The suggestion at the press conference a year ago was that a means of extracting energy from deuterium had been found. Persisting believers restrict themselves to more modest claims for phenomena which are, above all, not regularly reproducible. What has irretrievably foundered is the notion that cold fusion has great economic potential. The time has come to acknowledge that. It would be a cruel deception of a largely amused public not to admit that simple truth. And it would be a serious perversion of the process of science to obfuscate the failures of the past year by reference to the difficulties of measurement in an admittedly difficult field. □

More AIDS turmoil

The visa row surrounding this summer's AIDS conference may be a storm in a teacup.

THE US government has been painted into an uncomfortable corner on AIDS by its policy on visas for those infected with HIV wishing to attend the Sixth International AIDS Conference, planned for San Francisco in June. Formally, US law would exclude from the United States those who suffer from certain listed infectious diseases, or



even who are psychiatrically sick. HIV was added to the list only in 1987, on the basis of an amendment by Senator Jesse Helms.

The policy is not new. One of the memorable sights of the Western world is that of would-be permanent immigrants presenting to US immigration officers the X-ray films that show them to be free from tuberculosis, but even temporary visitors have to declare themselves free of that and other infections, AIDS included. It is ironical that some of those most concerned to learn at first hand where AIDS research stands now may be excluded from the conference in June. The International Aids Society, based in Frankfurt, is but the latest interested party to protest.

The issue is important, but should not be mistaken for a simple violation of the principles of academic freedom. International conferences at which attendance is forbidden to some on the grounds of race, nationality or politics do not deserve the name, or the support of serious academic societies. But the restriction that may affect those wishing to go to San Francisco in June is not in that sense discriminatory. Nor is there reason to suspect that the US immigration authorities will exercise in a discriminatory fashion the powers they enjoy, whose only rationale can be that the US population should be protected from unwanted infection. In any case, while the law as it stands is the law, there are also procedures by which those affected can get waivers, or otherwise be exempted from the restraints. The US Immigration Service says that it will be sympathetic to those who apply for waivers and exemptions. The time to complain will be when there is some evidence that it is responding otherwise.

It is more relevant that the annual AIDS conferences are in danger of getting out of hand. Last year in Montreal, as at the previous conference in Washington, the proceedings had an element of the circus. There are too many parallel sessions at which too many people have too much to say, and there are also sideshows, often provided by AIDS pressure groups, that have insistently raised the question whether there are not better ways than these of exchanging information on AIDS research. After Montreal, many serious researchers muttered that they would not return. The sponsors, notably the US National Institutes of Health, might usefully use the next few months to decide whether perpetuation is necessary. Serious people will not think they are turning their backs on AIDS if they decide to abandon the annual conference.

Meanwhile, there is the matter of visas for this year to settle. Fears that a few scores or even hundreds of people carrying HIV and visiting for a week or so would be a serious threat to the well-being of the United States are insubstantial. It would be different if there were thousands, and if they planned to stay for months. In short, there is no serious hazard in letting registered participants at the conference have visas, whatever their state of health. It would be a shame if the United States should spoil its excellent record on free access for the sake of a little bumbledom. □

Japan bashing pauses

Japan and the United States need a better mechanism for mutual stability than a stop-over at Los Angeles.

THE meeting of the finance ministers of the United States and Japan at Los Angeles last week-end seems to have done nothing to improve relations between the two most dynamic economies in the world. And even the earlier announcement that a deal has at last been struck on the export of US supercomputers to Japan will not blunt the edge of the continuing dispute (see page 370); the benefits to the United States and to Cray Research Inc. will not be as great as they have been made to seem. Yet neither the US Secretary of the Treasury, Mr Nicholas Brady, nor his opposite number, Mr Ryotaro Hashimoto, can relish the prospect of continuing backbiting. What is to be done?

The new development is the sudden collapse of the Tokyo stockmarket and the weakening of the yen, which has fallen in value against the US dollar by 7.5 per cent this month. Both developments probably have the same cause — the calculation by people outside Japan that their money would better earn its keep in the United States, where interest rates are higher and dividends more generous. But the trigger for the transformation has been the remarkable flight of money to West Germany, on the calculation that the prospect of reunification creates new profitable opportunities for investment. Japan is having to learn the hard way that the world in which it earns its good living is truly international.

There is nothing very remarkable about that. What is more surprising is that the United States appears to be indifferent to the dangers inherent in the developing situation. If the yen continues to fall (or even stays where it is), Japanese exporters will have their salesmen scouring the United States for orders in a trice, and the US trade deficit with Japan will worsen again. If, on the other hand, Japanese interest rates are increased so as to make investment in the yen more profitable, fewer Japanese will invest in US Treasury bonds and the difficulty of financing the US federal deficit will be increased.

In smaller, but locally no less painful, ways, the same troubles crop up elsewhere. In Britain, for example, even 15 per cent interest rates have not stemmed the decline of sterling, and may have to be increased further. Britain, fortunately, has a remedy which it has so far declined to adopt — membership of the currency stabilization mechanism of the European Monetary System; the British government is now talking up this idea more sympathetically than for the past year. What Japan and the United States now need is a club of the same kind to which they might belong, preferably one linked with the European system. What Mr Hashimoto was asking for at the week-end was the beginnings of such an arrangement. It is a great pity that he has been denied for not much better a reason than that Japan is due for — even in some sense deserves — a bout of misfortune. □

US Space Station needs another redesign

- Maintenance too heavy a burden
- Station essential for NASA's survival?

Washington

ONLY six months after a comprehensive restructuring of the US Space Station programme unleashed a torrent of criticism from Congress and from the project's international partners, more redesign work is on the cards. NASA (National Aeronautics and Space Administration) last week announced a review of plans for the \$30,000-million craft in response to an internal report which concluded that routine maintenance of the space station would require 2,300 astronaut-hours of 'space-walking' every year. In the 30-year history of the US space programme, astronauts have spent only 400 hours outside their craft.

The study, by astronaut William Fisher and NASA engineer Charles Price, surveyed the NASA contractors who are building the 5,578 parts that will reside on the outside the space station, and found that repairs and replacement of light bulbs, thermal blankets, electromechanical devices and batteries aboard the space station will require, on average, some external maintenance every day. Spacewalks, known in NASA parlance as 'extra-vehicular activity' (EVA), are considered risky because of danger from radiation and micrometeorites.

Fisher and Price asked the contractors to estimate a "mean time between failures" for each part. Then, calculating how long it would take to repair each part and allowing for a certain amount of overhead, the team arrived at an estimate of 2,284 hours of EVA time a year. That amount is based on an assumption of about one failure a day in a critical part on the outside of the station.

The estimated repair time for these failures at present stands at nearly four times the level NASA officials have set as their goal, a discrepancy Fisher and Price found "ominous".

If the maintenance requirements are not reduced, they conclude, space station astronauts would have to switch from construction to repair by the time the project was only 60-70 per cent complete. "That's unacceptable", Fisher said.

Responding to the results of the study at a meeting in Washington last week, NASA's associate director for space flight, William Lenoir, said that maintenance "has always been an issue lurking right beneath the surface. Now we're designing real hardware, not paper, and it was time to put in real numbers." But Lenoir played down the severity of the problem.

Saying that the estimates were "a snapshot in the middle of our study", he added "it's business as usual". Officials from NASA headquarters openly disputed the reliability of the Fisher-Price study, which came from the Johnson Space Center in Houston, Texas. At a press conference last Friday Lenoir characterized the study as a "worst case scenario" whose numbers were "not credible". But Fisher asserted



that the work was "a conservative estimate" and released a statement criticizing NASA for diminishing the study "simply because the results are unpopular." Whatever the magnitude of the problem, NASA said it would waste no time in finding a solution. Space station officials said that they would concentrate initially on finding the parts that are expected to consume the most EVA time and then either redesign them for greater reliability or relocate them so that they are accessible from within the pressurized crew compartments. "We're willing to spend money to make [a] part more reliable, or to bring [it] inside", said Lenoir: "we will probably

find that 80 per cent of the effort is allocated to 20 per cent of the items. We want to concentrate on those items." Another option is to use the station's two robots to make more of the repairs. But without costly upgrading, the robots as now configured are unlikely to be able to take over more than a quarter of the repair task. And adapting external parts to be more "friendly" to robotic manipulation will cost money as well, Fisher said.

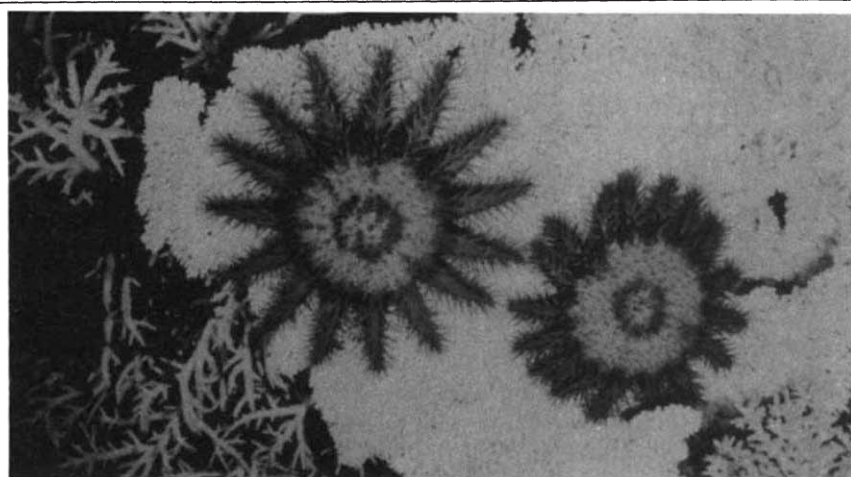
Nevertheless, Fisher said that robot technology is likely to improve far faster than spacewalking skills over the station's 30-year lifetime. Although "we may have to bite the bullet early on" by shifting emphasis to expensive and potentially troublesome robots, "the learning curve is steeper" for the machines, he said.

NASA administrators insist that the project's overall cost will not rise because of the changes. Lenoir said that the space station's current budget allows for some redesign. The preliminary final design is expected to be completed by the end of the year, although it is not known if the unexpected EVA problem will change the timetable. First in-space assembly of the station is scheduled to begin in 1995.

Another concern for NASA is how Congress will react to this latest problem with the space station. The congressional appropriations process is approaching its critical months and early responses from key legislators suggested that one solution might be to cut the space station's 1991 funding while NASA sorts out the problem. Three congressional hearings are scheduled this week on the EVA issue.

But analysts believe that although cutbacks are possible, cancellation of the entire project is probably not. "It's not the end of the line", says John Pike, a space analyst for the Federation of American Scientists. "I'm fairly confident that they can wrestle this problem to the ground. They'll have to; [the space station] is essential to NASA's institutional survival."

G. Christopher Anderson



Has the Great Barrier Reef survived the onslaught of the Crown of Thorns starfish? News from Australia, page 370.

Pleasing some of the people

Paris

A REPORT* just published by the UK House of Commons Foreign Affairs Select Committee, chaired by Foreign Office under-secretary Timothy Sainsbury, says there is "no reason why the UK should not rejoin" the United Nations Educational, Scientific and Cultural Organisation (UNESCO) in 12 months if there is "real evidence of improvement". Britain and Singapore left UNESCO in 1985, a year after the United States withdrew, all in protest at the policies of the previous director-general, Amadou-Mahdar M'Bow.

But since the beginning of March, when the committee heard evidence from Federico Mayor, UNESCO's present director-general, Mayor has announced reforms which have rocked the boat again.

A freeze on recruitment and the filling of vacancies angered some staff to the extent that they stopped work for two hours last week, complaining of a lack of consultation. In future, all new appointments will have to be approved by Mayor himself, while a new policy of decentralization will mean transfers abroad and fewer staff at the Paris headquarters. According to one source close to Mayor, most of the internal complaints were from members of a staff union appointed by M'Bow.

In addition, plans to appoint more than 20 senior programme co-ordinators and consultants have upset some US diplomats, who see them as going against earlier promises to cut costs. But according to estimates from within UNESCO, the new posts will cost about \$3.5 million over two years, while savings made through previous staff cuts and the new freeze have been estimated to reach \$18 million by 1993.

One appointment in particular has provoked criticism from US observers. As co-ordinator for communication and information, Mayor has chosen Henrich Yushkiavitchus, former deputy director of the Soviet State Commission on Radio and Television. The potential for censorship in the guise of information management was one of the chief issues that led to the US withdrawal from UNESCO. But, say those inside UNESCO, Yushkiavitchus has been behind efforts to liberate the Soviet media in recent years, and he is said to be liked by Western journalists.

Some of the reforms follow recommendations of an international commission chaired by Knut Hammarhjöld, and subsequent comments by a panel of experts headed by Peter Wilenski, in reports delivered to Mayor at the end of 1989. The number of UNESCO programmes has been reduced from 52 to 18, while the new co-ordinator posts are an attempt to place

talented individuals, sometimes brought in from outside UNESCO, in leading posts.

The new jobs are intended to achieve a more efficient management structure. In his policy document, Mayor launches an whole-hearted attack on the present administration. "UNESCO has become too rigid", he says, "a large slow-moving body, made up of staff too often weighed down by administrative tasks and who in many cases no longer work in the forefront of knowledge or of their own special field." He goes on to compare this structure to "a brain, proportionally reduced, with poor means of anticipation and orientation, and few, or no, alarm mechanisms".

According to one senior UNESCO official, the reforms were bound to attract

resistance. "If Mayor had announced these policies two years ago, everyone would have said 'bravo', he's a man of action. Leaner structures may be hard for some, but they are necessary."

Though the United States remains lukewarm, the new reforms have received a partly positive response in London. A spokesman for the Foreign Affairs Select Committee confirmed that the government would stick to its decision to "wait and see" over the next 12 months. The changes have gone "a long way" in terms of programmes, but are said to be "limited" in terms of administration, he said. Meanwhile, the new expansion plans seemed "uncertain and confusing", but, he added, "more time is needed to see whether the director-general can deliver".

Peter Coles

*UK Policy on UNESCO (House of Commons Paper 255. Vol. 1, 1989-90 Session.)

AGEING

Setting the memory norm

Washington

EARLY results from a study on memory loss in the inhabitants of half a dozen small Italian towns are providing data of unprecedented accuracy on rates of neurological deterioration. Project scientists hope that the survey will establish with far greater precision what "normal" memory loss is, in part to distinguish it from the early symptoms of Alzheimer's disease. Previous studies have suggested that between the age of 30 and 60, normal adults may exhibit a 60 per cent decline in name and face recognition.

The main sponsor of Progetto Memoria (Memory Project) is the Memory Research Centre of the Italian pharmaceutical company Fidia. The project is expected to establish baseline measurements of memory loss with statistical errors of as little as two per cent, an accuracy which Fidia scientists attribute largely to Italy's stationary population.

Because most people in the survey live in the place they were born, the scientists are studying an unusually homogeneous cross-section of the population, says Thomas Crook, president of the US-based Memory Assessment Clinic, which is participating in the project. Average rates of change in the population of the selected towns are as little as a few tenths of a per cent per year, Crook says. Most US populations are far more mobile, and less homogeneous, than their Italian counterparts.

"In the United States it's difficult to know who stayed and who left. Is the [remaining population] in a certain city the sickest or the best?", Crook asks. Although 5,000 Americans 50 years of age and older have had their memory tested by various methods in recent years, the

surveys have traditionally been tests of people who come to researchers with concerns about their memory. Such "samples by convenience" may exhibit systematic biases, says Crook.

Because Italy has a national health care system with comprehensive health records and a regularly-updated census, "the whole first step in standard epidemiology is done for us," says Francesco Grigoletto, a statistician from the University of Padua, Italy.

Progetto Memoria is travelling through Italy in a \$450,000 mobile testing laboratory. The scientists enlist local media and physicians to publicize the arrival of the team and to explain its objectives. Then the team places the mobile testing laboratory at the centre of each day's survey area.

Early results show that only 10 to 15 per cent of the selected population refuses to participate in the voluntary survey, and project scientists expect about 75 per cent overall participation by the end of the survey. Over 1,600 normal, healthy Italians between 20 and 79 years of age will be tested by the time the project is finished at the end of the year.

The survey uses tests specially designed to be independent of cultural, educational, or economic bias. In one test subjects are shown videotapes of between 2 and 14 people who introduce themselves and give some basic personal information, such as the name of their hometown. The participants are then shown the faces alone, and are asked to recall the correct names and personal details.

Other tests involve following a maze or recalling the location of everyday objects such as eyeglasses or keys.

G. Christopher Anderson

Eastern promise unfulfilled

Tokyo

ON the brink of a showdown over trade issues, the United States and Japan last week reached an agreement intended to increase sales of US supercomputers to Japan's universities and public research institutes. But although the new agreement may help diffuse trade tension between the two countries it is unlikely to have much effect on the actual number of US supercomputers sold in Japan.

The reluctance of Japan research institutes to buy US supercomputers has long been a cause a frustration in the US Congress. The US manufacturer Cray Research Incorporated dominates the world market but has only managed to sell two supercomputers to Japan's public sector. Nearly all government-financed purchases have gone to Japanese manufacturers such as NEC and Fujitsu.

In 1987 Japan agreed to make government procurement of computers more open, but so far this has not led to any new sales for Cray. Last year, supercomputers were listed as an item for possible trade retaliation under the US Omnibus Trade and Competition Act of 1988. But under the new agreement, worked out by trade negotiators in Washington last week, Japan has agreed to discourage "academic discounting", a practice whereby Japanese manufacturers sell supercomputers to public institutions at rock-bottom prices.

The discounts, often as much as 80 per cent of normal retail price, have effectively shut Cray out of the market. And government ministries have encouraged the practice by giving public institutions sufficient funds to buy supercomputers only at the highly discounted prices.

Government ministries will now allot bigger budgets for supercomputers, and will take into account performance as well as price when selecting computers. US and Japanese government officials welcomed the accord; failure to reach an agreement could have led to punitive trade tariffs against Japan under the Omnibus Act.

But the new agreement is unlikely to boost US supercomputer sales. Government ministries have increased their budgets for purchasing individual supercomputers simply by decreasing the total number of supercomputers which will be bought. In fiscal 1990, for example, government ministries will buy four supercomputers instead of the ten originally planned. And although Japan has promised to eliminate academic discounts, Japanese manufacturers have already reduced the retail price of their supercomputers, so making the discounts look smaller. As an official of Cray's Japanese subsidiary commented "the agreement in Washington is all very well but we will wait to see the results".

David Swinbanks

TELECOMMUNICATIONS

Battle over break-up of NTT

Tokyo

A BATTLE is developing over the future of Japan's domestic telecommunications giant, Nippon Telegraph and Telephone Corporation (NTT).

Earlier this month, the Telecommunications Council, an advisory body to the Ministry of Post and Telecommunications, recommended the break-up of NTT into three separate companies, one to handle local services, one for long-distance telecommunications, and a third for mobile telecommunications, such as car telephones (see *Nature* **341**, 474; 1989).

The council's plan is intended to promote competition but there is strong opposition to the council's proposals in government, industry and political circles.

The recommendation to break up NTT comes on the heels of a review of NTT by the Management and Coordination Agency that calls on the company to reduce its staff and allow fairer competition with newcomers in the telecommunications market.

Three new domestic telecommunications companies, Teleway Japan Ltd,

Japan Telecom Company and Daini-Denden Inc., have welcomed the council's recommendations. The new companies compete with NTT in the long-distance market but have to use NTT lines for local connections. As a result, NTT still controls 98 per cent of the market and long-distance charges in Japan remain very high compared with those in other developed nations.

NTT management and unions are united in their opposition to the calls for its break-up. NTT president Haruo Yamaguchi seems prepared to discuss separation of the small mobile telecommunications sector but is otherwise strongly opposed to the plan. He says a break-up would interfere with NTT's plans to introduce a new nationwide telecommunications network, ISDN, and would result in reduction of NTT's research and development activities.

The powerful federation of Economic Organizations (*Keidanren*) has called for a three-year delay in making any decision on the future of NTT. And the opposition Japan Socialist Party (JSP) is also against the plan. But the most powerful opponent

Have the starfish gone for good?

Sydney

REPORTS that the Great Barrier Reef off Australia has survived the latest outbreak of Crown-of-Thorns starfish and is on the mend are premature, marine biologists here say. A survey, carried out by the Australian Institute of Marine Sciences (AIMS) between November last year and January, reported significant reductions in the numbers of Crown-of-Thorn starfish (*Acanthaster planci*) between Lizard Island and Innisfail, in the centre third of the reef. On one of the worst-hit reefs, off Green Island, an estimated 1.5 million starfish during the height of the outbreak in 1980 reduced coral cover by 20 per cent over five years. The AIMS survey reported noticeable regrowth in the area.

But despite widespread press reports that the reef is no longer under threat, the AIMS survey reported reduction in starfish numbers only in the central third of the reef, between Cooktown and Townsville.

On Old Reef, near Townsville, seven starfish were reported in 1985, 11 in 1986, 3,266 in 1987, 1,000 in 1988 and only two in 1989. However, south of Townsville there are increasing reports of outbreaks of starfish. In 1985 only 5 per cent of the reefs in this area reported infestations. Four years later there were outbreaks on 40 per cent of the reefs.

What happens to the starfish once they have run out of food is a mystery. There is no evidence of mass mortality and there has been little research so far into the movement of larvae, believed to be the source of the outbreaks further south.

It seems to take 15 years for reefs to recover from Crown-of-Thorns infestations, although slow-growing corals can take up to 100 years to recuperate. The cause of such outbreaks is still unknown and continues to be a subject of debate between those who think the infestations are man-made, with overfishing removing the natural predators of the starfish, and those who believe the outbreaks to be a natural phenomenon. Tania Ewing

of the plan is the Ministry of Finance.

The ministry has been selling off government-held shares of NTT since the company was privatized in 1985. The share sales are an important source of government revenue and the government still holds 5 million shares worth over ¥6 million million (\$40,000 million) at the current market price (¥1.23 million). But the share price has been plummeting and the ministry fears that break-up will cause a further fall in value. Nevertheless, the Ministry of Post and Telecommunications seems determined to adopt the council's recommendations this month, and a fight over the future of NTT seems inevitable.

David Swinbanks

Cut unkind to chemistry

London

THE chemistry department at Royal Holloway and Bedford New College (RHBN), announcing that it will take on no new students this October, has become the latest casualty of the continuing financial crisis in British universities. Students who had already accepted places are to be offered alternative courses at RHBN or referred to other institutions. Once existing students leave, only three or four staff will remain to teach chemistry to geology and biochemistry students. Ironically, RHBN is one of five sites in the University of London earmarked for an expansion of science teaching.

Like many of London's colleges, RHBN is accumulating a financial deficit,



in its case at the rate of £1 million per year. It has floated several ideas to raise funds, including selling part of its art collection, and building high-quality housing on its botanical gardens.

Closing the chemistry department will make only a small dent in RHBN's deficit, but the decision was swayed by the expectation that the Universities Funding Council (UFC) will finance the necessary early retirements. If cuts were made elsewhere, RHBN principal Professor Dorothy Wedderburn says, the UFC would accept no responsibility.

The department was chosen by the UFC for restructuring following a nationwide review of university chemistry and physics departments under the UFC's predecessor, the University Grants Committee (UGC). It was concluded that departments should have more than 200 full-time undergraduate students to be viable. In general, the UFC has not forced changes upon small chemistry departments, but the University of London's senate was informed in October last year that the UFC would accept no new chemistry students at RHBN unless the department could move above the 200-student level. Wedderburn believes the department's

relatively poor research rating with the UFC influenced this action.

The chemistry and physics review was one of a series pursued by the UGC in the late 1980s, which resulted in several department mergers and closures. The UFC has now abandoned these reviews, and has instead introduced a competitive bidding system for student places (see *Nature* 342, 843; 1989). Universities must say by June next year how many students in each subject they wish to teach in 1994–95, and student numbers will be allocated by the UFC from 1991–92 according to these figures.

In theory, this should mean that universities may plan their own long-term strategies, and not have rapid changes forced upon them such as those to which RHBN has succumbed. But the new system is beset by uncertainties. Continuing deficits may force some institutions, to make cuts, and the bidding system itself is far from clear.

The UFC circular letter which explained the system said that the allocation of student places would depend partly on "offer prices" made by universities for the cost of each place. But financially stretched universities are worried that they may not be able to make competitive offers, and will suffer accordingly.

Understandably, the UFC's vague assurance that it would "not expect to ask any university to face a rate of contraction of resources which it could not manage", has done little to reduce these fears.

In contrast, the bidding system introduced by the Polytechnics and Colleges Funding Council for 1990–91 funding guaranteed support at 95 per cent of the previous year's level, with competitive bidding only for the last 5 per cent.

Peter Aldhous

STUDENT LOANS

Universities to take more of the strain

London

THE British government is amending the student loans bill to compel universities to help implement the controversial scheme. This follows the refusal of university vice-chancellors to cooperate any further than required by law (see *Nature* 344, 1; 1 March 1990).

Universities must confirm that students are eligible for a loan, but are not yet, as was feared, required to check on students' financial references. Department of Education and Science officials say they will discuss any arrangements to reimburse universities' additional administrative costs with the Committee of Vice-Chancellors at a future date.

Peter Aldhous

Lab's future uncertain

London

IN a strongly worded letter to Ron Coleman, chief scientist at the UK Department of Trade and Industry (DTI), 300 staff members at the DTI's National Engineering Laboratory (NEL), in East Kilbride, Scotland, demand urgent action to define the laboratory's future. Uncertainty over the NEL's role and shedding of staff, the letter says, "are preying on us like a cancer". In common with several other government-financed laboratories, the NEL is to be reconstituted as a 'government agency', with more freedom to manage its own finances. But the NEL's case is unusual in that agency status is designed as an intermediate step towards an eventual sell-off to private investors, probably in the mid-1990s.

The government's reorganization policy is to give NEL and other laboratories an 'arm's-length' relationship with parent departments, but the letter claims that the DTI has shifted plans for the NEL "from week to week". One NEL researcher says "I'm not sure if there is a master plan", and the letter says that staff cuts threaten "whole areas of research", which include the development of renewable energy sources, alternatives to chlorofluorocarbons (CFCs) and computerized manufacturing.

Since privatization plans were first announced almost two years ago, the NEL's staff has been cut from 620 to 525, with further reductions to about 400 planned for the near future. DTI will support the laboratory in the short term (£11 million over the next three years, compared with about £20 million a year at present), after which the NEL must exist entirely on its research contracts. There will be no forced redundancies, according to the DTI, but the staff say that in the absence of detailed official information, "rumour runs rife". They also complain that the NEL's local management has been "emasculated" by the failure to appoint a chief executive for the new agency since the post was advertised at the beginning of the year.

The letter to DTI arose as a spontaneous action after a meeting of NEL staff, but has since been backed by the largest union at the laboratory, the Institution of Professionals, Managers and Specialists (IPMS). IPMS says the loss of staff at the NEL will jeopardize the laboratory's ability to compete for external research contracts (which account for about 20 per cent of total income). It questions the wisdom of selling a laboratory running "strategic research", such as work on energy technologies, to the private sector.

Peter Aldhous

FOSSILS

Lizzie not out of the woods

London

A SURPRISE donation of £10,050 will aid the appeal by the National Museums of Scotland (NMS) to keep 'Lizzie', the 340-million-year-old fossil of the earliest-known reptile, in Scotland where she was discovered. The grant comes from the Geologists' Association (GA), and brings the total amount raised to £120,780. The GA have welcomed the opportunity to contribute to the appeal to secure what its press spokesman Eric Robinson of University College, London, calls "one of the most significant fossil finds of all time" in Edinburgh.

The target is £207,000, which must be raised by the end of July to prevent Lizzie's sale by her discoverer, professional collector Stan Wood, to the Museum für Naturkunde in Stuttgart. Apart from £104,000 pledged by the NMS (see *Nature* 343, 398; 1 February 1990), the GA grant is the biggest single donation to the appeal fund.

NMS curator Ian Rolfe says that many people have expressed a wish to contribute, ranging from individuals to large corporations: potential benefactors should get in touch with him at the Royal Scottish Museum in Edinburgh.

Most of the GA membership is English, and the decision to help secure Lizzie for Scotland may well raise some eyebrows. Robinson dismisses this: Lizzie was alive

long before England or Scotland were invented, he says. Wood found the fossil in the Carboniferous limestones at East Kirkton, along with a rich haul of fossil flora and fauna. The purpose of the grant is to keep Lizzie as the centrepiece of this important collection, not because she happens to be Scottish.

A precedent for the GA's action exists: it gave financial aid to Bristol Museum to help curator Peter Crowther buy an ichthyosaur fossil that became the focus of an exhibition on marine reptiles last year (see *Nature* 338, 381; 1989). The GA stepped in to prevent the specimen, offered for sale by a private collector, from being sold to a buyer in Japan.

The deliberations over Lizzie's fate have raised wider issues of the export of geological specimens (see *Nature* 343, 106; 11 January 1990). Tam Dalyell, Member of Parliament for Linlithgow — the area of Scotland that includes the East Kirkton limestones — demanded of Richard Luce, the Minister for the Arts, what measures the government planned to aid the rescue of what he described as "my constituent, Lizzie" (*Hansard*, 13 March 1990, page 170). In reply, Luce had recommended that the Reviewing Committee on the Export of Works of Art should consider how its criteria might be amended to encompass geological specimens in future.

Henry Gee

SCIENTIFIC ETHICS

Harvard legislates on conflict

Boston

AFTER a year of study and a month of vigorous debate, the Harvard Medical School last week set out its new conflict-of-interest rules for faculty and researchers.

Under pressure from researchers at some of the school's fourteen affiliated hospitals, measures that would have made illegal a number of practices, as recommended in the earlier controversial proposal, have been omitted (*Nature* 344, 97; 1990). But, pending approval next month by the affiliated hospitals' governing body, a committee will be established to oversee industry-university relations at institutions affiliated with the medical school.

Under the new rules, royalties on published work and honoraria will be routinely allowed as before, but a category of links between researchers and industry will now be "allowable only after disclosure, approval review, and oversight". Activities requiring oversight will include consulting for a company while simultaneously evaluating one of its products in a clinical trial, holding equity in a company providing research support and presenting research results without dis-

closing financial interests in a company whose product is the subject of research.

The new guidelines also delineate activities that will be specifically permissible provided they are fully disclosed. These include conducting or participating in a clinical trial of a technology invented by the researcher or serving on the board of directors of a business from which the faculty member receives research support.

Daniel C. Tosteson, dean of the medical school, terms the guidelines a "significant change" and emphasizes their necessity if university faculty members are to retain their "credibility as seekers after truth". He says the new guidelines will "promote technology transfer" while keeping researchers "from slipping into an inappropriate relationship with industry".

Barbara J. McNeil, head of the school's department of health care policy and chair of the committee that initially developed the conflict-of-interest rules, says she is "extremely pleased" with the school's adoption of the new measures. But how the oversight committee will work, she acknowledged, "remains to be seen".

Seth Shulman

PRIZE NEWS

Tyler award for chemical messengers

THIS year's \$150,000 Tyler prize for environmental achievement is to be shared by Thomas Eisner and Jerrold Meinwald of Cornell University for their studies of chemical ecology — the science of how organisms interact chemically using 'messenger substances' to attract, defend or reproduce. In a collaboration that has spanned more than 30 years, Eisner, a biologist, has shown how insects communicate, while Meinwald, an organic



Eisner (left) and Meinwald.

chemist, has defined this 'communication' in chemical terms. Their chemical discoveries have led to the characterization of an array of compounds, some of which have led to advances in pest control and the development of drugs to treat human diseases.

Eisner has championed the cause of conservation, calling species extinction the "silent calamity of our time". In 1970, Meinwald co-founded the International Centre of Insect Physiology and Ecology in Nairobi, Kenya, to study ways to control the spread of disease to humans and livestock by ticks and tsetse flies.

D. G.

Immunology and development

THE Fifth Louis Jeantet Prize for Medicine has been awarded to developmental



biologist Nicole le Douarin, from Nogent-sur-Marne in France, and immunologists Harald von Boehmer and Gottfried Schatz, whose research centres on



Douarin (top), Schatz (left) and Von Boehmer.

cell organelles. Von Boehmer and Schatz are based in Basle, Switzerland. The laureates share 2.1 million Swiss Francs, to be presented on 6 April. Schatz will use the money to employ a young researcher, the other prizewinners concentrating on new research equipment.

P.A.

Influenza according to Hoyle

SIR—Perhaps we should examine more closely the possibility that a primary extraneous source of influenza and other viruses is terrestrial ocean water, with or without polar ice and other solid matter, carried up into terrestrial or solar orbits, including pseudo-cometary orbits, by grazing collisions of large celestial bodies impinging upon the Earth. In general, the type of solar orbit would be a stronger function of the trajectory of the grazing body than that of the Earth, but the case of major interest would be a trajectory in the Earth's orbital plane.

The entrained water would be rapidly frozen in the near-vacuum and its viruses might well survive storage times of the order of 10^8 years, as neither mainstream solar radiation nor solar flares might be expected to be a strong agent in their destruction. Comets survive. If the Earth crossed the wake of the debris, however, both ambient atmospheric conditions and special conditions brought about by solar-flare activity might well play a part in enhancing the probability of non-destructive release of the viruses. Because the latter would most probably be pre-human, the human race would have had no opportunity to develop resistance. It would not be necessary to postulate an extraterrestrial origin for the viruses.

D. G. ANDREWS

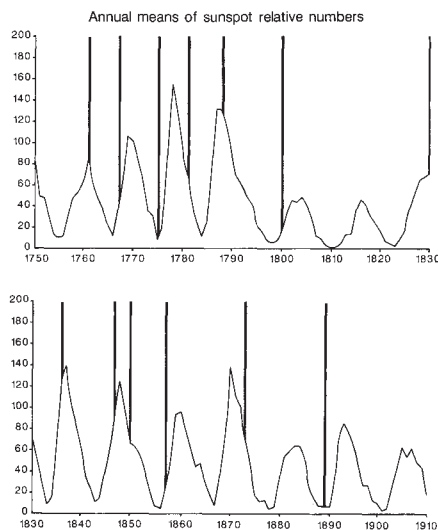
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SIR—In their letter repeating the idea of a connection between sunspot maxima and influenza pandemics, Hoyle and Wickramasinghe¹ have correctly pointed out that "past experience has shown that false correlations of phenomena with the sunspot cycle may look good over a few cycles but go seriously adrift after an appreciable number of cycles".

The graph below shows sunspot cycles and influenza outbreaks using Hoyle's own list for pandemics and for sunspot maxima (except for 1767 which was not a maximum year), together with the annual sunspot numbers published by Waldmeier². It seems obvious that the occurrence of pandemics is fairly well distributed over all phases of the solar cycle — contrary to what the authors want to imply.

Furthermore, with the exception of the pandemic of 1889 (coinciding with a sunspot minimum), it is doubtful which of the historic epidemics listed by Beveridge belong to the category of pandemics. For comparison, Creighton's *History of Epidemics in Britain*³ looks rather different: 1562, 1580–82, 1665, 1733, 1743, 1782, 1833, 1847, 1889. Among these years, the

phase of the sunspot cycle before 1700 is uncertain: however, the years 1733, 1743, 1782, 1833 and 1889 all happen to be years of sunspot minima (3 cases) or one year before minimum (2 cases).



As an astronomer, I refrain from strengthening my point by biological arguments. These were recently published by Henderson *et al.*⁴. More information on the history of epidemics and pandemics may be found in *Basic and Applied Influenza Research*⁵.

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4. Henderson, I.M., Hendy, M.D. & Penny, D. *J. theor. Biol.* **140**, 289–303 (1989).
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No carbon tax

SIR—I am disturbed by the suggestion of a so-called carbon tax on fuels, for several reasons.

(1) When taxes are introduced for non-fiscal reasons, they end up benefiting only the Exchequer, with the original reason long forgotten. The road fund is perhaps Britain's best known example.

(2) Carbon dioxide is not a pollutant, but an essential ingredient of the Earth's atmosphere. In geological time, the amount of carbon dioxide in the atmosphere has varied very widely, and although the reasons for the widest variation are known, the details are mysterious and complex. There may be natural influences of which we know nothing that are either increasing or

decreasing the atmospheric content. An increase in carbon dioxide may even be beneficial by stimulating plant growth.

(3) The reason advanced for taking steps to lower the carbon dioxide content, or at least to slow its rate of increase, is the so-called greenhouse effect. It is not known whether this effect is occurring, and, if it is, whether this would be bad or good. The tendency of the climate over the next period of time may be towards another ice age, in which case the greenhouse effect might be of the greatest value to us.

(4) The proposed carbon tax would be a great burden to manufacturers, public and private transport and to householders wishing to use any form of power in the house. Indeed, it is the proclaimed object of the tax to cause a change in the power consumption habits of the world.

Habits are not so easily changed, and this proposal is in the style of many directives that have proved unsuccessful during the past century. A more likely result is that habits would continue largely unchanged, and that the extra burden would end up in inflation, which is more likely to cause damage to the world and its economy than is carbon dioxide.

Finally, I wonder why we are so convinced that the present state of the world is the best possible one. In the past, carbon dioxide concentration, temperature and sea level have all varied far more widely than are envisaged in any of the projections made for the greenhouse effect. The result of all these changes has been the world we know, which is apparently regarded as satisfactory. Why then should any changes be regarded as necessarily deleterious?

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Whistle-blowing

SIR—You are correct in pointing out (*Nature* **343**, 396) that "whistle-blowing is always a dangerous trade, the more so when directed against more powerful colleagues", and we share your regret that "*Nature* is not in a position to offer protection of any kind to those exposed". Should matters be allowed to rest there? It is hardly likely that such problems are confined to the Indian subcontinent. The Council for Science and Society is gathering material on this topic; we would welcome accounts of particular incidents (which would be treated in strictest confidence) and also suggestions for means of protecting whistle-blowers in the future.

JEROME R. RAVETZ (Chairman)

BERNARD DIXON (Vice-Chairman)

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The embarrassment of cold fusion

David Lindley

The variable and transient claims of experimental evidence for cold fusion made a moving target which attracted too much enthusiasm and too little derision.

THOSE readers who consider that this journal has published too much on 'cold fusion' should be grateful for what they have been spared.

In the year since Stanley Pons and Martin Fleischmann made their famous announcement at a press conference at the University of Utah, we have received a barely imaginable quantity of letters — most of them respectably typed, one or two handwritten entirely in capital letters — attesting to remarkable latent powers of creative thinking by scientists around the world. For only a few of these offerings was it thought necessary to consult expert reviewers; fewer still survived that scrutiny.

Of the fraction of papers submitted that have thus reached print, the account appearing this week (see page 401) is of special significance. Michael Salamon and his colleagues, like the teams from Yale University and Brookhaven Laboratory, from the California Institute of Technology, and from the UK Atomic Energy Authority Laboratory at Harwell, have searched for nuclear emissions (neutrons, gamma-rays, electrons and protons) from cold fusion cells, and found nothing. The difference is that Salamon works at the University of Utah, and the cold fusion cells he examined were in the laboratory of Pons and Fleischmann.

This is not quite the same as saying that what Salamon *et al.* looked at were Pons and Fleischmann's cold fusion cells. As the article makes clear, Pons will not concede that any of the electrolytic cells were, at the time they were being examined, in fact producing anomalous heat. There was a two-hour period when, according to Pons, a cell under examination was producing excess heat — but at that time the detectors and computers of Salamon and his colleagues were not working, having been put out of action by a power cut.

To this, Salamon *et al.* provide a clever response. Any neutrons emitted by the working cold fusion cell would have created some secondary radioactivity in the neutron detector, which should have been measurable once the equipment was switched on again after the power loss. But no such secondary activity was found, which allows Salamon *et al.* to conclude that no conventional fusion reactions could have produced the claimed excess heat.

The tidy summary of their paper is therefore that during a five-week period which came some time after the press

announcement of Pons and Fleischmann on 23 March last year, cold fusion cells which may or may not have been producing excess heat certainly produced no anomalous nuclear emissions.

In the light of the known history of cold fusion, one has to ask whether this news will change the terms of the debate.

During the past year, the original claims of Pons and Fleischmann have diminished; the experimental evidence has been subtracted from not added to. In their 'preliminary note' (*J. electroanalyt. Chem.* **261**, 301–308; 1989 and erratum **263**, 187–188; 1989) Pons and Fleischmann said that they had found, as well as excess heat, production of tritium in the cell, and the emission of neutrons, detected by characteristic secondary gamma-rays from the surrounding water-bath. But the tritium concentration in a working cell turned out to be only three and a half times greater than that in the original stock of heavy water. Critics were quick to point out that such an increase is consistent with a recognized difference in the electrolysis rate of deuterium and tritium on account of their different masses.

Later, Petrasso (see *Nature* **339**, 183; 1989) argued that the gamma-ray signals presented by Pons and Fleischmann as evidence of neutron emission had the wrong characteristics to be genuine detections, and were more likely to be instrumental artefacts.

This left only the claims of excess heat,

which were themselves challenged at the Baltimore meeting of the American Physical Society (see *Nature* **339**, 4; 1989).

Pons and Fleischmann must be given credit for declaring from the outset that the amount of excess heat they saw was too great, by orders of magnitude, to be consistent even with the levels of nuclear emission that they described in their preliminary note.

Supporters of heat production by cold fusion have always said that some new physical process, by which deuterons can fuse in secret without giving off tell-tale nuclear by-products, was indicated by their results.

Thus arises the schism that separates those who believe in cold fusion from those who put the whole episode down to wishful thinking. On one side, non-believers see the negative results of Salamon *et al.* as incontrovertible proof that nothing unusual is happening; on the other, believers take the same results as affirmation that cold fusion indeed demands new physics, which they knew already.

The two sides are separated by a matter of faith, not one of science.

Efforts to bridge this divide have been made all the more difficult by the unceasing refusal of Pons and Fleischmann to say clearly and fully what their experimental evidence for cold fusion is. In the wake of their preliminary note and its published errata (which were themselves an extended version of a preliminary list of errata handed out as a photocopied sheet with the 10 April 1989 copy of *Journal of Electroanalytical Chemistry*), there came rumours (subsequently and vehemently denied at the Los Angeles meeting of the Electrochemical Society on 8 May last year) of heat generation by cells containing ordinary water rather than heavy water, and of the production of ^4He in significant amounts. The discovery of ^4He in the gases coming from a cold fusion cell was briefly trumpeted as a demonstration that deuterons absorbed into the palladium electrode were indeed fusing, but by a reaction that produced ^4He and a gamma-ray rather than tritium and proton or ^3He and a neutron, as conventional nuclear physics would have it.

Inconveniently, however, materials scientists pointed out that if helium had been formed in the palladium electrode, it would have stayed there; the correct test would have been to look for helium in the palladium itself, not in the gases evolved

IMAGE
UNAVAILABLE
FOR COPYRIGHT
REASONS

Early optimism: Pons (left) and Fleischmann with large-scale model of their 'cold fusion' flask at their press conference. (AP)

by the cell. But because Pons and Fleischmann had given out the suggestion about helium production in their usual teasing and informal way, it could be retracted without much pain on their parts. During April and May last year, there were constant assurances that a detailed account of their experimental methods and results was in preparation, would be properly submitted to a scientific journal, and would appear during the summer, or in the autumn — or perhaps a little later.

But what was reprehensible a year ago has now become absurd.

Still there are whispers of a hundred-page manuscript, replete with facts and figures, which the world will soon see. Most of the world, sadly for Pons and Fleischmann, is unlikely to care, except perhaps out of historical curiosity and a desire that the tale be neatly ended.

The waxing and waning of these various pieces of ancillary evidence, not to mention the sporadic nature of the successes achieved even by expert cold fusion researchers, was a wonderful liberation to those who rummaged through their undergraduate physics textbooks in search of forgotten phenomena that could be adapted into theories of cold fusion. It was a boon too that solid-state physics and quantum mechanics conspire to offer such a variety of unexpected and counter-intuitive effects to the eager enquirer.

If the ideas submitted to this journal are a representative sample, theories of cold fusion generally have the same logical structure as the assertion: "If we had some ham, we could make a ham sandwich, assuming there's some bread handy". Starting from some bona fide physical effect, one argues that if it were many orders of magnitude more important than it actually is, cold fusion would be possible; left unspoken is the assumption that the effect in question is of some relevance in palladium saturated with hydrogen.

In the proliferation of theoretical proposals, there were surprisingly few basic ideas. A number of early suggestions referred to the Oppenheimer-Phillips effect: at very low energies, two deuterons in close proximity tend to orientate themselves so that their constituent neutrons approach each other and keep the protons apart, which enhances the fusion rate because it permits the neutrons to interact while minimizing the electrostatic repulsion of the protons.

Explanations of this sort illustrate a common feature of cold fusion theories. The discrepancy between the standard fusion rate and what was needed to generate the heat seen by Pons and Fleischmann amounts to some 60 orders of magnitude, and the Oppenheimer-Phillips enhancement is a matter of ten per cent or so; nevertheless the reader was invited to agree that things were moving in the right

direction, and accept that a bit of numerical fine-tuning would be needed to take care of the details.

A somewhat more sophisticated clutch of ideas emerged as people latched on to the idea of effective masses. Two deuterons bound together in a molecule have a tiny but calculable chance of fusing and if, as a thought-experiment, the mass of the orbiting electrons is increased, the molecule becomes more tightly bound and physically smaller, and the fusion rate rises. It was then noted that electrons in palladium can have effective masses several times greater than that of a free electron; if two deuterons could be bound together with one of these 'heavier' electrons, an interesting fusion rate would result.

But a little learning is a dangerous thing. Electrons in a metal obtain their effective masses because they are not bound to a single atomic site, but move in concert throughout the lattice: push against one, and you push against them all. But if one of these lattice electrons is detached from the system so it can bind two deuterons into a molecule, it is no longer part of the electron collective, and reverts to having its normal mass. In other words, the effective mass of an electron in a solid depends on what you are doing to it, and if you want it to bind an isolated molecule, it can no longer have a high effective mass.

The third broad category of cold fusion theories rested on more sophisticated uses of collective effects in solid-state physics.

The general drift was that deuterons, being particles of zero spin, could fall into a Bose-condensed state, in which their wavefunctions were all identical and periodic throughout the lattice. (This condensation can occur only if the temperature of the system is less than a few degrees above absolute zero but, as with the Oppenheimer-Phillips mechanism, it was the principle that mattered.) Once in this condensed state, one deuteron could interact with another at a different lattice site in the palladium, because all the identical periodic wavefunctions of all the deuterons would have their maxima at the same places.

These theories had the special attraction that they could easily be decorated with the jargon, at once forbidding and enticing, of solid-state physics: Bose-condensates, Bloch states, Wannier functions... like the Paris fashions, they outface mockery.

Nevertheless they were all wrong, and for another straightforward reason. The fusion rate for two deuterons is calculated from their two wavefunctions, multiplied by the nuclear interaction rate. The latter is a very short range force; only at separations of a few nuclear radii is the nuclear reaction rate significant. The only important contribution to the fusion rate, there-

fore, comes from the product of the wavefunctions when the deuterons are very close. But the wavefunctions in the Bose-condensed state are calculated explicitly by ignoring the nuclear interactions; they are valid everywhere except at close range. Where the assumptions that lead to Bose-condensation are correct, the fusion rate is negligible; where the fusion rate might be significant, the Bose-condensation model is wrong.

All cold fusion theories put forward so far can be demolished one way or another, but it takes some effort. Although cold fusion was, in terms of 'ordinary' physics, absurd, it was not obviously so; it contravened no fundamental laws of nature. This made it easy for advocates of cold fusion to insinuate that some arcane but genuine phenomenon of quantum-mechanical solid-state physics might provide a credible theoretical foundation, and likewise made it impossible for sceptical physicists to give any clear and general proof that cold fusion could not work; all they could do was rebut one daft idea after another, and even that required a good deal of patient explanation.

But it has to be said that one of the reasons that Pons and Fleischmann prospered early on was that few people were willing to stand up and say why they thought cold fusion was nonsense. (One or two physicists suggested in private that it was up to this journal to take on the task in its editorial pages). After the 23 March press announcement, the response of most experts consulted by science reporters was academically correct but journalistically weak. It's a very interesting idea, was the gist of the scientific community's opinion, and we really can't say what we think of the experiments until we've seen more details, or tried it for ourselves, or consulted our colleagues in the chemistry department. This measured scepticism, contrasted with the unhesitant declarations coming from Pons and Fleischmann, sounded like academic nose-holding, as if physicists knew they had been beaten but could not bring themselves to admit it.

Perhaps science has become too polite. Lord Kelvin dismissed the whole of geology because his calculations proved that the Sun could be no more than a few million years old; Ernest Rutherford is still remembered for his declaration that talk of practical atomic energy was "moonshine" — but the stature of neither man has been noticeably diminished by their errors, which were as magnificent as their achievements. Kelvin and Rutherford had a common-sense confidence in the robustness of their judgements which the critics of cold fusion conspicuously lacked. Would a measure of unrestrained mockery, even a little unqualified vituperation, have speeded cold fusion's demise? □

David Lindley is an Associate Editor of Nature, based in the Washington office.

Möbius and problems of inversion

Who says that the theory of numbers is strictly academic? An old theorem due to Möbius has unexpectedly proved to be a way of solving physical problems of inversion that may have important applications.

THE belief that pure mathematics is only fortuitously useful is widely shared, even by mathematicians. So why is the practice of science increasingly mathematical? There are two explanations. First, good luck; mathematicians do so many things that some of them must be useful. Second, psychology; the purest mathematicians have an unconscious sense of the urgent problems of the real world, and shape their interests accordingly.

There is also, of course, the Hilbert phenomenon. David Hilbert (1863–1943), whose first claim on public attention was his demonstration that Euclid's axioms are not the self-consistent structure they had seemed for two millennia, afterwards bent his talent to the solution of physicists' problems, founding a tradition now carried on by people such as Atiyah (Oxford) and L. D. Fadeev (Moscow).

But there may also be something in the view that all mathematics is potentially useful, that mathematicians have littered the literature with gems of technique whose usefulness is waiting to be discovered. For did not the nineteenth century's preoccupation with continued fractions prove useful, if briefly, in field theory? Did not Hamilton's quaternions, devised as a means of completing the algebra of vector quantities by the definition of a quotient, turn out to have interesting connections with the spinor algebra of relativistic quantum mechanics?

That is the spirit in which one should celebrate the use now discovered for a recondite contribution to the theory of numbers by Möbius (1790–1846), best known for the topological conundrum called the Möbius strip. Among other things, Möbius noted a simple inverse relationship between functions in number theory, which takes the following form.

First, take some function $f(n)$ of the integer variable n and another function $F(n)$ defined as $\sum f(d)$, where the summation runs only over the divisors of n , 1 and n included. Then, according to Möbius, it is possible to invert the functional relationship into the simple $f(n) = \sum \mu(d) F(n/d)$ where the sum again runs over all divisors of n , and where the coefficients $\mu(d)$, all either zero or ± 1 , reflect the prime composition of d .

Briefly, $\mu(1) = 1$, and $\mu(d) = 0$ except when d is either a prime number or a composite number which is the product of, say, r distinct primes, when it has the value $(-1)^r$. One can while away hours on

aircraft journeys verifying that the inversion works. To show that $\sum \mu(d)$ differs from zero (with the same restrictions on the summation) only when $n=1$ requires more ingenuity; one has to express n as a formal product of prime numbers and then show that the sum is $(1-1)^n$.

What use is this? Inversion is the key word. Physical problems are most often inversion problems — inferring the velocity profile in the Earth's crust from seismic signals, for example, or the linear distribution of interplanetary electron density from the measured Farady rotation of the plane of polarization of a radio signal from a satellite of some kind. And now Nan-xian Chen, from the Technical University at Beijing, has turned Möbius's inversion theorem to practical use by the exercise of more than a little ingenuity (*Phys. Rev. Lett.* **64**, 1193; 1990). The work was done when Chen was at the International Institute of Theoretical Physics at Trieste.

The trick is to show that the integers in Möbius's inversion formula can be replaced by continuous variables, which hangs on a proof that the infinite series arising do indeed converge. The essence of Chen's paper is the proof that if $A(\omega) = \sum B(\omega/n)$, where the summation extends from $n=1$ to infinity, the inverse is given by $B(\omega) = \sum \mu(n)A(\omega/n)$, with the same summation rule.

Chen proves his point by demonstration, with a string of examples only otherwise solved with difficulty. One of them, a new result, is neat enough at least to illustrate the potential power of the method. Suppose that the vertices of an infinite one-dimensional lattice are all occupied with interacting atoms, so that any one of them experiences a potential $V(x) = \sum v(nx)$, where the summation in n is from 1 to infinity and $v(x)$ is the elementary pair-wise interaction. How, one might ask, can that be related explicitly to $V(x)$, which might be measurable? What the inversion gives is simply $\sum \mu(n)V(nx)$ or, more explicitly, $V(x) - V(2x) - V(3x) - V(5x) + V(6x) \dots$

A more interesting example is that of how it may be possible accurately to infer the frequency distribution of the vibrational states of a solid lattice, say $g(v)$, from measurements of specific heat at constant volume. Apart from numerical factors involving Planck's constant and Boltzmann's constant, the latter is simply

the integral from zero to infinity of $v^2 g(v)$ weighted by an appropriate Planck-Boltzmann factor allowing for the increased excitation of higher frequencies with increasing temperature. At low temperatures, it is usually feasible to represent the measured specific heat as a power series in the temperature T , beginning with a cubic term.

As if pulling a rabbit out of a hat, Chen relates $g(v)$ directly to the measured coefficients in the power series representing the specific heat. As a reminder that the theory of numbers lies at the basis of all this sleight of hand, values of Riemann's zeta function for integral multiples of 2 appear throughout, which means that they can be written as Bernoulli numbers. Chen's other example is that of inferring the temperature distribution of a composite black body from measurements of its power output, which he says is a problem of current interest in remote sensing.

Where will all this lead? The ideal, for Chen, would be that somebody should put a previously unsolved problem through the new Möbius mill. It will be interesting to see which problems first suggest themselves as candidates. A more demanding question is whether it will be feasible to extend the trick to problems that are not simply one-dimensional. On the face of things, that might seem a mere formality, but it takes only a little scribbling to run into problems essentially tied up with the multiple connectedness of all but one-dimensional spaces. But that should not be a discouragement; rather, a challenge. It is fair to guess that, with Chen's proof that even Möbius has something to tell the modern world, a small army will now be scouring the literature of the theory of numbers in the hope of finding other useful tools in what may have been unjustly regarded as a backwater.

There is no shortage of material. Chen himself quotes the fifth edition of the classic *Theory of Numbers* by G. H. Hardy and E. M. Wright (Clarendon, Oxford; 1979), which does indeed tell all about the Möbius inversion without hinting that it may have physical application. Alongside that is a neat proof that Ramanujan's sum, defined as $\sum e^{2\pi i h m/n}$ with the sum in h running only over values less than n and prime to it, is also $\sum \mu(n/d)d$, where d is both a divisor of m and n . Surely, one is bound to ask, there must be some value in that?

John Maddox

More to muscle than MyoD

Miranda Robertson

THREE years ago, Harold Weintraub and colleagues published¹ the arresting claim that the introduction of a single gene, *MyoD*, into mouse fibroblasts could induce the fibroblasts to differentiate as muscle. They concluded that *MyoD* encodes a master-regulator for skeletal muscle differentiation and stimulated much subsequent research as a result of which the striking simplicity of the original picture has been largely lost. Thus on page 454 of this issue, Schäfer *et al.*² report that the introduction of *MyoD* into liver cells is not sufficient to induce the expression of muscle-specific genes, which can however be activated if the transfected liver cell is then fused with a fibroblast. The implication is that the master-regulatory activity of *MyoD* is in fact slave to other proteins which suppress it in liver cells but assist it in fibroblasts. This is consistent with what is now known about the structure and action of *MyoD*.

Myogenic family

The master-regulator theory in its purest form was first shaken by the discovery that *MyoD* is one member of a family of at least four closely related proteins that are expressed in skeletal muscle cells and activate the myogenic programme in fibroblasts³⁻⁵. All these proteins activate their own transcription in transfected cells^{4,6}, as might be expected of a protein responsible for the maintenance of a differentiated state; and they also activate one another's transcription. How or whether this interacting set of positive feedback loops operates *in vivo* is not known, but the intervention of repressors of some kind may be deduced from the fact that not all of them are expressed in all muscle cells at all stages of development.

A possible source of regulatory influences resides in other related proteins: the myogenic protein family belongs to a larger family, known as the helix-loop-helix (HLH) family after a 60-amino-acid structural motif deduced from their N-terminal sequences⁷, and which specifically bind DNA. This should not be confused with the classical helix-turn-helix motif of the prokaryotic DNA-binding proteins: in the HLH proteins the DNA-binding function is thought to reside in a basic region adjacent to the helices, which are believed to be a dimerization motif.

The 60-amino-acid HLH region on its own is sufficient to activate muscle-specific genes in fibroblasts⁸, in the absence of the carboxyl-terminal region whose function is unknown. The interpretation of this fact is however complicated by the ability of proteins of the *MyoD*

family to activate their own transcription: thus the HLH region may suffice to activate the endogenous myogenic proteins but not necessarily to execute the entire programme.

Specific binding sites for *MyoD* have been demonstrated upstream of several muscle-specific genes⁹⁻¹¹, and it seems likely that the myogenic proteins play a direct part in the induction of the muscle phenotype by activating them. Specific binding of *MyoD* (or its cousins) to DNA cannot in itself however explain the tissue-specific expression of skeletal muscle genes. (This should not surprise: see refs 12 and 13.) The myogenic proteins share the HLH motif with at least ten other proteins^{7, 14-16}, including four that bind to immunoglobulin gene enhancers in B lymphocytes and four *Drosophila* proteins, mutations in which disrupt development. Of these, one of the *Drosophila* proteins and two of those that bind immunoglobulin enhancers (E12 and E47) will bind specifically *in vitro* either as homodimers or as heterodimers with *MyoD* to the κ E2 DNA motif in the immunoglobulin light-chain enhancer⁷. This immediately raises questions about the basis of tissue specificity. Both the immunoglobulin light-chain enhancer and the identified *MyoD* binding sites contain the same consensus sequence; and E12 and E47 are present in most cell types. Why, then, are not muscle-specific genes activated in all cells by E12 and/or E47? And why are immunoglobulin light-chain genes silent in muscle? Clearly, as Murre *et al.*⁷ acknowledge, specialized cells must contain an array of positive and negative regulators that modify the actions of transcriptional activators. This is what the experiments of Schäfer *et al.*² with transfectant heterokaryons have demonstrated in a general way.

The conclusion reached by Schäfer *et al.* was in fact implicit in an earlier paper from the Weintraub laboratory¹⁷. It is a legitimate criticism of the master-regulator interpretation of the original experiments that 3T310T^{1/2} fibroblasts, like muscle, are of mesodermal origin and can differentiate spontaneously into myoblasts in culture. Weintraub *et al.*¹⁷ therefore transfected a number of non-mesodermal cell types with *MyoD*, and reported that many of these also could be induced to express muscle-specific genes. But not all of them ceased to express the specialized genes of their original tissue; and one kidney cell line and two human liver-tumour cell lines could not be transformed at all. Weintraub *et al.* attributed this failure to the presence of inhibitors, or the absence of positive regulators, but did not allow it

to influence their conclusion that *MyoD* is sufficient to activate the myogenic programme.

Schäfer *et al.* have chosen a different emphasis, and have pursued the implication of positive and negative regulators that modify the activity of *MyoD*. Their argument rests on the effects of the relative numbers of nuclei contributed by the liver and the fibroblast parents to the multinucleate heterokaryon that results from their fusion. According to their observations, muscle-specific genes are more often induced when the number of fibroblast nuclei in the heterokaryon exceeds the number of *MyoD*-producing liver-cell nuclei. From this they infer that the positive effects of the additional *MyoD* contributed by the transfected liver nuclei are counteracted by a corresponding increase in endogenous inhibitors produced by the same nuclei. Nor, they argue, can the effects of the inhibitors be overcome by increasing the amount of *MyoD* alone: even liver cells expressing very high levels fail to express muscle-specific genes until they are fused with fibroblasts. The need for positive factors contributed by the fibroblasts could account for this.

Regulating the regulator

Meanwhile, a detailed mutational analysis of *MyoD* and two other helix-loop-helix proteins by Davis *et al.* in Weintraub's laboratory has shown in principle how tissue-specific positive and negative regulation might work¹⁸. They have established that the helices are essential for the formation of heterodimers between *MyoD* and other HLH proteins, and that substituting the *Drosophila* T4 helical or loop region for the same region of *MyoD* does not affect its ability either to bind DNA or to activate muscle genes. Mutations or exchanges in the adjacent basic region can however affect both DNA binding and (independently) the activation of the myogenic programme. Thus a mutant in which the *MyoD* basic region is disrupted

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can no longer bind DNA; and one in which the E12 basic region is substituted for the MyoD region can still bind DNA but no longer activates muscle genes. Davis *et al.* suggest that the six amino acids that differ between E12 and MyoD in this region are required for interactions with a transcriptional activator that recognizes MyoD only when it is bound to DNA in a muscle-specific enhancer.

MyoD mutants in which the basic region no longer binds DNA prevent specific DNA binding by the wild-type protein, presumably by forming mutant-wild-type heterodimers that can no longer bind DNA (the binding affinity of the wild-type monomer being insufficient on its own). This suggests a possible mechanism for inhibition of the activity of MyoD in cells that do not respond to transfection. Davis *et al.* cite the unpublished identification of a helix-loop-helix protein that naturally lacks a basic region and

suppresses the myogenic action of MyoD *in vivo*, and speculate that the regulated production of isolated dimerization motifs may be one way of controlling the actions of transcriptional activators that can bind DNA only as dimers.

These ideas may serve as suggestions for how myogenic activation might be regulated in principle. In practice, muscle-specific enhancer regions are large and complex, and contain binding sites for many regulatory proteins as well as MyoD; and of course MyoD exists in its complete form and not as the 60-amino-acid helix-loop-helix domain used by Davis *et al.* As Murre *et al.* ominously remark⁷, a complete understanding of the regulation of muscle-specific gene expression may elude biologists for some time. □

Miranda Robertson is Biology Editor of Nature.

GALAXY EVOLUTION

Making ellipticals by mergers

Joshua E. Barnes

AN elliptical galaxy is recognized by its smooth, nearly featureless oval shape and by the characteristic way in which its surface brightness declines with radius. This profile is known as the 'de Vaucouleurs' or ' $r^{1/4}$ ' law after G. de Vaucouleurs, who in 1948 noticed that the logarithmic surface brightness of an elliptical falls off linearly when plotted against the fourth root of distance from the centre of the galaxy. On page 417 of this issue¹, Wright *et al.* report that several starburst galaxies (which, as the name implies, have been stimulated into vigorous activity) imaged in the near infrared also follow a de Vaucouleurs law. These observations support the idea that some ellipticals are forming in the present epoch as a result of mergers between disk galaxies².

In photographs taken with visible light, starburst galaxies are irregular systems, laced with chaotic patterns of dust. Most are involved in interactions with other galaxies. Those studied by Wright *et al.* are an extensive class, in which two or more galaxies have coalesced to form a single object festooned with tidal tails and other debris³. Yet emissions in the near infrared (wavelengths around 1 μm), radiated largely by stars formed well before the merger, reveal little evidence of such turmoil. Evidently, these old stars have already relaxed into a distribution typical of an ordinary elliptical galaxy.

Numerical experiments have shown that a good approximation to an $r^{1/4}$ law profile can be set up in a distribution of stars subjected to a violently fluctuating gravitational field^{4,5}. One way to generate such violent relaxation is to merge

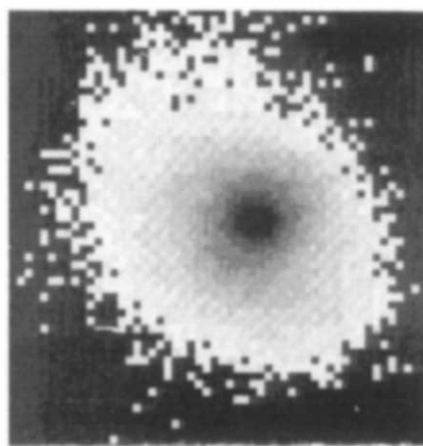
equally matched disk galaxies; if these disks are embedded in massive dark haloes, the resulting remnants resemble ellipticals not only in their luminosity profiles but also in their shapes and dynamics⁶. The new observations, as well as Schweizer's study⁷ of the merger remnant NGC7252, indicate that the old stellar populations of merging galaxies respond as expected.

However, the galaxies studied by Wright *et al.* are unusual in another respect — they emit prodigious amounts of far infrared radiation (wavelength 8–1,000 μm). Indeed, one of them, Arp 220, is among the most luminous far-infrared sources known. All of the galaxies

contain not only the stellar wreckage of a pair of disks, but also significant amounts of hot dust, molecular gas and young stars. This material is concentrated towards the centres of these merger remnants⁸, where it seems that as much as 10^{10} solar masses of molecular gas may be efficiently converted to stars⁹. So, whereas the overall shapes of these merger remnants are dominated by older stars, the central regions are being laid down 'before our eyes' in an episode of violent star formation.

The brightest of these systems seem to derive most of their far-infrared luminosity not from star formation but from an active galactic nucleus shrouded in hot dust⁸. There is extensive observational evidence that interactions, and especially mergers, can provoke such nuclear activity, although there is little theoretical understanding of the mechanisms that transport material from a few hundred parsecs (the scale of the nucleus) down to scales ten orders of magnitude smaller where it can be violently consumed by a massive black hole. If the nuclear activity persists, it may burn off the enshrouding dust and gas to reveal a central source of nonthermal radiation. The power levels and number densities of the ultraluminous infrared galaxies are such that they may well be the immediate progenitors of low-redshift (recent) optical quasars⁸.

Quasars were much more numerous at higher redshifts, $z \approx 2$. Likewise, a large fraction of elliptical galaxies is found in rich clusters, where there has been little merging since cluster formation, presumably at redshifts $z \geq 1$. The tempo of merging should have been significantly higher in early epochs when galaxies were closer together; the galaxies involved may have contained larger fractions of gas than those at present. Nonetheless, observations and theoretical models indicate that mergers between present-epoch disk



Arp 220, one of the starburst galaxies observed by Wright *et al.*¹. Left, optical (blue) photograph (30×30 arcminutes) dominated by the effects of dust associated with extensive star formation. Note particularly the dark streak of dust running across the face of this galaxy. (Courtesy J. Mazzeella and J. R. Graham.) Right, near-infrared photograph rendered as a negative and set against a dark background (30×30 arcminutes). In contrast to the optical photo this image is much more regular, with brightness declining smoothly from the centre to the edge of the galaxy. (Courtesy G. S. Wright.)

galaxies are producing remnants with structural properties similar to cluster ellipticals. This similarity suggests — although it does not prove — that the mergers we observe at $z < 1$ may be much like the events responsible for the majority of elliptical galaxies.

If so, observations like those presented by Wright *et al.*¹ have a significance extending beyond the specific galaxies they chose to study. These remarkable objects may be the low-redshift analogues of proto-elliptical galaxies at higher redshifts, or 'living fossils' providing glimpses of occasional rapid transformations of galaxies. High-resolution observations of nearby ellipticals may soon tell us what fraction have quiescent quasars lurking in their cores; deep infrared and optical studies will constrain the tempo of merging and they will help relate local conditions to those prevailing at earlier

times; detailed numerical models could unravel the mechanisms responsible for starbursts and nuclear activity. In short, there are good reasons to think that we now possess the means to understand the formation of elliptical galaxies. □

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GROWTH FACTORS

Growth without inflation

T. Michael Dexter and Harry White

ALTHOUGH appreciable efforts have gone into characterizing growth inhibitory molecules, the work has met with relatively little success, in part because of the problems in designing suitable assay systems where specific cytostatic, rather than cytotoxic, effects can be rapidly and reproducibly measured. On page 442 of this issue¹, Graham *et al.* have used a well designed assay system to identify a cytokine, macrophage inflammatory protein 1 α (MIP-1 α), which is produced by macrophages and is able to inhibit proliferation of primitive haemopoietic cells (putative stem cells). This cytokine may well turn out to be an important component of the growth regulatory network for haemopoietic cell production.

In regenerating tissues, such as bone marrow, skin or intestinal epithelia, the cells produced are just sufficient to replace the effete cells lost as a consequence of damage or ageing — supply and demand are finely balanced. Mature cells are continually produced from a small population of stem cells that are laid down during embryogenesis. In the haemopoietic system (which can be used as a model for other regenerating tissues), stem cells can differentiate in many ways to produce developmentally restricted progenitor cells. These then proliferate and develop into mature blood cells. During normal 'steady state' haemopoiesis, production of the required numbers of blood cells is achieved mainly through amplification of the committed progenitor cell population, most of the stem cells being in a proliferatively quiescent state². Following a cytoreductive insult, however, stem cells are rapidly recruited into

cell cycle, undergo self-renewal and differentiation, and the haemopoietic system recovers to normal levels. How, then, is this homeostatic balance maintained?

In the past decade, much emphasis was placed on the role of growth-stimulating molecules in regulating cell production³. Indeed, some of these molecules have already reached clinical trials and are showing obviously beneficial effects⁴. A question that has vexed biologists, however, concerns the regulatory response following a cell growth stimulation. Is the production of stimulator switched off? Or, are molecules produced that can counteract the effect of the growth stimulus? Likely candidates for inhibitors include the so-called 'chalones', which are naturally occurring molecules found in the tissues on which they act, and which have selective, reversible and non-toxic growth inhibitory properties (see ref. 5 for a review).

These principles of negative feedback are not new, but controversy surrounds this whole area of research. Nonetheless, the proliferation inhibitor characterized by Graham *et al.*¹ has most of the characteristics of a 'chalone': it is produced by mature haemopoietic cells (macrophages), and selectively inhibits proliferation of primitive multipotential haemopoietic cells but not proliferation of developmentally restricted progenitor cells.

The apparent specificity of MIP-1 α for haemopoietic cells may become blurred as other systems are investigated. Consider, for example, the effects of LIF/HILDA/DIA^{6,7}, a cytokine that facilitates differentiation of some leukaemic cell lines, sup-

ports the growth of other cell lines and inhibits differentiation of embryonic stem cells. Transforming growth factor TGF- β is another agent well known for its ability to promote or inhibit differentiation, or to stimulate or retard cell growth, according to the cell type^{8–11}. It will be intriguing to unravel the molecular mechanisms behind the different effects that can be achieved by the same cytokine.

How does MIP-1 α relate to other molecules that inhibit proliferation of haemopoietic stem cells? Frindel and colleagues¹² have described a tetrapeptide that prevents resting G₀ stem cells from entering the cell cycle; although this peptide was thought to be specific for stem cells, an inhibitory effect on committed progenitor cells has been reported¹³, and its precise role remains an enigma. In addition, TGF- β has profound differentiation-linked growth inhibitory effects directly on haemopoietic cells *in vitro*^{14,15}. Like MIP-1 α and the tetrapeptide, the effects of TGF- β seem to be cytostatic rather than cytotoxic. Recent results from our laboratory may well have a bearing on how these molecules work *in vivo* to regulate stem cell proliferation: we find that the response of stem cells to MIP-1 α is rapid (within hours), although the response to TGF- β is slow (more than 24 hours) (J. Hampson, C. Heyworth and T.M.D., unpublished observations). Perhaps the speed at which the brakes are applied can vary in different physiological situations?

This raises the question of the part these cytokines play in regulating stem cell proliferation in the bone marrow. Stem cell proliferation is regulated at a local level in all regenerating tissues, so control is unlikely to be mediated by circulating molecules. But as the MIP-1 α molecule was originally cloned from activated macrophages¹⁶, is it released from such cells into the general circulation? Not necessarily: many examples exist of potent growth stimulatory molecules (such as the interleukins IL-2 or IL-3) that are released from T lymphocytes *in vitro*, but which probably exert their effects locally *in vivo* by direct cell contact. These are not normally present as circulating molecules. Also, Lord and co-workers¹⁷ have shown that the stem cell inhibitor SCI (almost certainly equivalent to MIP-1 α) is produced by macrophages showing a distribution in the bone marrow corresponding to sites of decreased stem cell proliferation¹⁸. TGF- β is also present in the bone marrow and could potentially also exert local effects.

The idea, then, is that in normal steady-state haemopoiesis, local production of growth inhibitory molecules ensures that few stem cells are called upon to proliferate. Indeed, stem cell growth inhibitory molecules can be detected in the bone marrow in such circumstances¹⁷. When the

STELLAR EVOLUTION

stem cell population is reduced, the relative level of inhibitor falls and a potent growth stimulus is produced; as stem cell levels recover, the situation is reversed and inhibitor again takes over. How is this achieved? The answer is apparently in the stem cells themselves, which produce a factor or factors to inhibit the production of the growth stimulus¹⁷, thereby limiting the size of their own population.

Self-limiting feedback regulation can also be found in the mature haemopoietic cell compartments. Apoptosis, or programmed cell death, will occur in factor-dependent haemopoietic cell lines in the absence of growth factor¹⁹. Such observations suggest that steady-state levels of mature haemopoietic cells could be actively regulated by the availability of haemopoietic growth factors that are essential to their survival³.

The growth inhibitory effects of MIP-1 α and other cytokines on haemopoietic cells encourage their application in the treatment of disease. Reduction of normal haemopoietic stem cell proliferation during administration of cell-cycle-specific cytotoxic drugs to cancer patients could alleviate the bone marrow toxicity of chemotherapy. Another possibility would be to use stem cell inhibitory molecules in conjunction with chemotherapy to treat leukaemias, some of which have well characterized cell cycle regulatory defects²⁰, and might, therefore, fail to be protected by the stem cell inhibitor. These approaches can now be explored, and hold much promise for clinical intervention in the future. $\square$

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Lumps in planetary nebulae

Charles Alcock

AMONG the more beautiful celestial phenomena (widely available on astronomical posters) are the planetary nebulae. These are translucent shells of gas which surround very hot, white-dwarf-like stars (and have nothing to do with planets; the name is an unfortunate historical misnomer). The shells are generally 0.1–1.0 parsecs (pc) in radius, and the emitted light is largely due to emission lines from ions of the common atoms. Many of these nebulae contain small ($<10^{-3}$ pc) condensations. Dyson, Hartquist, Pettini and Smith¹ now suggest that these cool, dense gas clouds had their origin in maser-emitting lumps near the progenitor star. This

not a good physical model of the superwind itself. What is it that drives mass loss at such copious rates from red giant and supergiant stars? Theoretical efforts have so far failed to provide a convincing mechanism. It is clear that the pressure of radiation acting on the dust grains found in this circumstellar material can drive the outflow; however, the surfaces of the stars are too hot (above 2,000 K) for grains to exist. Clearly, some mechanism has to lift some material off the star, where it can cool and dust can form. At that point, radiation pressure can finish the job. What Dyson *et al.* have shown is that the dense condensations



The Helix Nebula, NGC7293, in the constellation Lyra. Dyson *et al.* show that condensations in the nebula probably formed early in the star's evolution and would have been apparent as SiO masers during its superwind phase. (Photo by David Malin.)

idea might eventually help elucidate the mechanism of formation of the planetary nebula itself.

There is now overwhelming circumstantial evidence that planetary nebulae result from the 'superwinds' observed from red giant and supergiant stars, where mass is lost at rates up to 10^{-4} solar masses per year. Once the superwind has stripped the bulky envelope from the star, the exposed core is compact, very luminous, and apparently drives a brief period of high-velocity, low-mass-loss wind. This latter wind evacuates a spherical bubble inside the still-expanding superwind material, which therefore begins to appear shell-like. Finally, ultraviolet photons ionize the superwind material, producing the characteristic planetary nebula.

This phenomenological description is perfectly adequate, except that there is

seen in one particular nebula, NGC7293, were probably formed near the star during the superwind phase. (The condensations do not seem to have resulted from a thermal instability in the present nebula.) They establish that the material is cold, and probably molecular. Further, they demonstrate that these clumps would have survived the superwind phase and the subsequent high-velocity-wind phase. Finally, Dyson *et al.* propose that these clumps, when they were very close to the parent star, were SiO maser clumps.

Bright maser emission is characteristic of a large fraction of all stars during the superwind phase. Maser emissions from many of the low-lying rotational transitions in excited vibrational states of SiO molecules are commonly observed. Radio-interferometric measurements by Moran

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*et al.*² of the SiO masers in a couple of these stars (VX Sagittarius and R Cassiopeia) showed that the maser emission came from about a dozen 'hot spots' near the surfaces of the stars. Ross and I³ then showed that the gas densities in these maser regions had to be much higher than the mean density in the outflow. The gas appeared to be highly clumped. Dyson *et al.* have identified the clumps seen in NGC7293 with the SiO maser clumps observed in the superwinds.

This identification is highly plausible, as the authors have shown. The important question now is: does this help us understand the superwind itself? This depends on the answer to an interim problem. Are these clumps peripheral to the superwind dynamics, or are they central to the process? If it turns out that the formation of the lumps is central to the origin of superwinds, then Dyson *et al.* have made a very useful contribution to this interesting field. I think it will turn out that the primary mechanism of mass loss involves the initial ejection of dense lumps from

these stars according to a mechanism originally proposed by Salpeter⁴, in which patches on the stellar surface cool temporarily, allowing local dust formation. Radiation pressure on the dust will do the rest.

Further progress in this field will probably result from further analysis of the kind carried out by Dyson *et al.* It seems increasingly likely that the mass outflow in the superwind phase is highly complex, and not readily modelled using the traditional assumptions of spherical symmetry and stationarity. More effort on understanding the phenomenology must precede more theory. □

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DEVELOPMENTAL BIOLOGY

Compartments in vertebrates?

Peter A. Lawrence

DEVELOPMENTAL compartments in insects are so well accepted now that it has become *passé* to discuss them. Several recent papers, in particular one by Fraser, Keynes and Lumsden on page 431 of this issue¹, will move them back into the spotlight, for what is known of insects now looks as if it may be true of vertebrates as well. We know that insects are made piecemeal; thus, groups of founder cells are designated early in development and, from then on, all the descendants of the cells of a group construct a particular piece of the larva and adult called a compartment. The compartments have precisely the same borders in each individual, but within those borders the founder cells generate clones of diverse sizes and shapes that differ from one individual to another². The founder cells are selected by the positions they occupy, not by ancestry. Thus, if a cell is marked before founder cells are allocated, the clone it generates will sometimes span the compartment border, whereas a cell marked at, or after, founder cells are defined will produce descendants that always remain confined within the compartment. The prime evidence for compartments depended on cell-marking experiments, which showed that both the segment boundary^{3,4} and boundaries within the segment^{5–7} act as lineage restrictions; after the founder cells are allocated, clones of cells remain confined to one side of a boundary or the other.

The link with genetics, which has

become such an important part of modern thinking on insect design, was proposed and developed by Garcia-Bellido, Ripoll and Morata^{8–10}, who suggested that the developing compartment would be co-extensive with the realm of action of a specific class of regulatory or 'selector' genes^{8–10}. The selector genes would determine the pattern made and the developmental pathway^{9,10} followed by each compartment. Thus the compartment theory envisages the animal as being made in modules, each growing and shaping itself independently of the others and each being subject to somewhat independent genetic control. The key piece of evidence for the theory was the observation that the wild-type *engrailed* gene of *Drosophila* is required in all the cells of every posterior compartment and in none of those belonging to anterior compartments; posterior cells that are mutant for *engrailed* develop anterior properties¹¹.

To decide whether there are developmental compartments in vertebrates² it is important to agree on diagnostic criteria for a true compartment. Historically, the first evidence was cell lineage, for the analysis of clones scored a line of demarcation across the wing in an incontrovertible manner, a line that clearly divided the set of cells in the wing and its thoracic segment into two subsets^{6,7}. But this evidence alone is not enough, for there is the possibility that some inconsequential aspect of the way the organ is constructed — such as cells coming

together that originated apart — might create the impression of a compartment and gain developmental significance under false pretences¹². So I now think evidence from genetics and/or molecular genetics is needed as well. In insects the different states of determination of cells in the various compartments are signalled by the requirement for, and expression of, homoeotic genes such as *engrailed* or *Ultrabithorax*. Some of the compartments defined originally, such as the dorso-ventral compartments in the wing, do not meet both criteria; although clearly identified by cell lineage, they do not (yet?) have genetic correlates¹³.

There is also an important misconception about compartments. Perhaps because we always used to emphasize that compartment borders need not coincide with anatomical landmarks, the idea has gained currency that genuine compartment boundaries must be invisible: witness this definition: "A compartment is a region whose boundaries, unlike those of a segment, cannot be seen by simple inspection"¹⁴. Not so: compartments are defined primarily by cell lineage and secondarily by the expression of regulatory genes — there is nothing in our understanding of them that requires that they be visible or not. What one can see will depend on the methods available. For example, in the milkweed bug *Oncopeltus*, although the segment boundary and the parasegment boundary are lineage restrictions and, unusually, both are marked by changes in cellular pigmentation that are visible to the naked eye, only the segment boundary coincides with a change in cell shape³. It has recently been shown that both boundaries can be defined at cellular resolution by mapping the expression of *engrailed*¹⁵. Other compartments are so obvious that they have been known for decades; the lineage segregations in embryos that are traditionally called germ layers may include several examples⁷.

During the past 15 years I have gained the impression that workers on vertebrates have preferred to regard compartments as evidence that insects are odd beasts with quirky developmental strategies of their very own. Their prejudice was strengthened by the results of Jacobson and colleagues, who performed cell-marking experiments on *Xenopus*^{16,17}, but failed to persuade others that there might be developmental compartments in amphibia.

Things have changed, however, and the evidence for compartments in the chick is growing. The argument initially derived from the expression of regulatory genes with respect to rhombomeres. Rhombomeres are metameric units in the hind-brain of vertebrates which are demarcated by grooves; the neurons in each mature at different times and send out axons in

different patterns¹⁸. Although the reality of rhombomeres was the subject of fierce debate at the beginning of the century¹⁸, there is now no doubt about their significance, mainly because regulatory genes are expressed in specific sets — for example *Krox-20*, a zinc-finger gene, is transcribed in rhombomeres 3 and 5 (ref. 19), whereas *Hox-2.9*, a homoeobox gene, is expressed only in rhombomere 4 (refs 20, 21). (See ref. 22 for a recent discussion on rhombomeres and homoeobox genes.)

Until now, there was no information on the cell lineage of rhombomeres, but on page 431, Fraser, Keynes and Lumsden present a compelling argument that, taken together with the molecular genetics, suggests rhombomeres are compartments *sensu stricto*. They show that, before a certain time, a marked cell in the embryonic hindbrain will generate a clone of dispersed cells that can populate more than one rhombomere; after that time, the marked cell produces descendants that, although they mingle with other cells, do not cross rhombomere boundaries. The stage when rhombomeres become determined as a specific set of founder cells coincides with the time when the rhombomere boundaries can first be seen as a series of ridges. Even so, the exact cellular interface between one compartment and the next has not yet been defined and the

lineage relationships between cells of the neural tube proper and of the ventral floor plate are still somewhat unclear. And there is still an important test to be done: a compartment is a specific set of cells defined by cell lineage and it is necessary to find out if the boundary of that set coincides cell-by-cell with the boundary of expression of regulatory genes such as *engrailed* or *Hox-2.9*. Coincident lineage tracing and antibody staining will probably be the way to do this.

The *engrailed* gene is crucially involved in the establishment and maintenance of insect compartments — what are the roles of *engrailed* homologues in vertebrates and how do they relate to rhombomeres? These questions can now be approached with a monoclonal antibody that recognizes a conserved epitope of some 11 amino acids in the *engrailed* homoeodomain²³. Patel *et al.* argue that this is a key component of the molecular signature of the *engrailed* gene and its homologues, allowing the mapping of *engrailed* expression in several different phyla²³.

In insects, *engrailed* is expressed in posterior compartments, but in vertebrates the *engrailed* homologue is found in a band of cells that overlaps parts of both the midbrain and hindbrain²³, which is where the rhombomeres develop¹⁸. In insects, the first compartments are parasegments, single metameres that form first and then are divided up with the help of the *engrailed* gene²⁴⁻²⁶; by contrast, in vertebrates the *engrailed* homologue appears to be expressed in a block before the delineation of rhombomere boundaries^{18,23}. The new antibody will help us to understand the reason for these apparent

differences, as well as to discover whether the boundaries of expression of the *engrailed* homologue coincide with any rhombomere boundary.

The source of metameres in vertebrates and insects may even be a common segmented ancestor. This assertion is suggested by a set of astonishing facts: the antennapedia and bithorax complexes in insects have their homologues (by homoeobox and other sequence comparisons) within the *Hox-2* complex of vertebrates. The order of these genes on the chromosomes and the spatial order (head-tail) of their domains of expression is conserved between the corresponding genes in arthropods and vertebrates^{27,28}. The new findings of Fraser *et al.*¹ increase the common ground, for it now seems that rhombomeres, like parasegments, are units both of homoeobox gene expression and cell lineage.

It is significant that the definition of the rhombomere compartments in vertebrates, by analysing both the growth of clones and the expression patterns of selector genes, could not have occurred without the basic research on insects^{2-11,29,30}. It is not the first time that work on experimentally amenable organisms has illuminated fields thought to be impossibly remote. I hope that opinion — that "sovereign mistress of effects" — will change and the relevance of studies on molecular and developmental genetics of flies and worms will come to be better appreciated by workers on vertebrates. □

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MEMBRANES

Violating the one-way system

John Armstrong and Graham Warren

THE shelves of pharmaceutical companies are said to be cluttered with thousands of chemicals, natural or synthetic, that failed to fulfil their potential as antibiotics, weedkillers or miracle cancer cures. Because much of modern biology owes its origins to the analysis of the effects of such molecules, it would not be surprising if hidden among the cobwebs were compounds of great interest to cell and molecular biologists. The antifungal antibiotic, brefeldin A, is one such molecule. Two recent papers in *Cell* from Lippincott-Schwartz *et al.*^{1,2} do not exactly turn the conventional view of traffic between organelle membranes on its head, but they do seem to make it face backwards. Specifically, they relate the action of brefeldin to two other highly topical issues, the relationship between microtubules and the membranes of the secretory pathway, and the existence of a hitherto elusive com-

partment between the endoplasmic reticulum and the Golgi complex.

Brefeldin has been known for over 30 years. Under the alias of decumbin, it was originally isolated from a strain of *Penicillium decumbens* responsible for spoiling corn and generating an aroma "reminiscent of exaltone"³. As dutifully reported in *Nature*³, the material was best prepared after growing the *Penicillium* in "a broth prepared by steaming 200gm. of peeled diced potatoes in 500ml. of tap water for 30 min." (ref. 3). Structurally, brefeldin bears a passing resemblance to the prostaglandins⁴, but considering its activity against unicellular eukaryotes such as *Candida*⁵, it is hard to imagine it acting as either a hormone agonist or antagonist.

The first indication that brefeldin affected the secretory pathway was that it caused the G protein of vesicular stomatitis virus, a popular workhorse for studying mem-

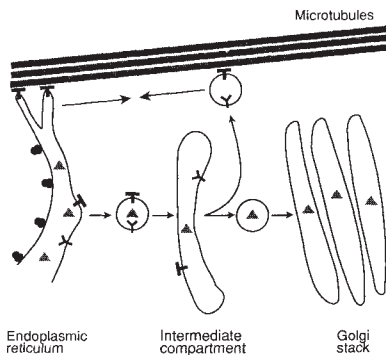
brane traffic, to accumulate in a high-mannose form characteristic of the endoplasmic reticulum (ER), and prevented it from reaching the cell surface⁶. Thus transport from the ER to the Golgi complex had apparently been blocked, but by a mechanism quite distinct from other anti-Golgi drugs such as the ionophore monensin⁷. Lippincott-Schwartz *et al.*¹ and Fujiwara *et al.*⁸ then produced an important clue as to why transport to the Golgi no longer occurred: the Golgi had disappeared. Marker enzymes of the early stages of the Golgi complex underwent rapid and spectacular redistribution in the presence of brefeldin and ended up in the ER, a step backwards on the secretory pathway. No Golgi complexes were visible in treated cells and, perhaps most impressively, the protein ERp99, a member of the 'KDEL' group of resident ER proteins⁹, had acquired resistance to endoglycosidase H, an indication of exposure to carbohydrate-modifying enzymes that are more usually found in the Golgi. Lippincott-Schwartz *et al.* explained these results by postulating that cells normally have transport pathways not only from the ER to the Golgi, but back again: by analogy to vesicle traffic in the nerve axon, they referred to these routes as 'anterograde' and 'retrograde' transport respectively. Normally the anterograde pathway would be the more rapid, causing net secretion of proteins. Brefeldin, however, would specifically block this route, allowing the retrograde pathway to predominate. Apart from the pedantic point that it is difficult to describe a route between compartment B and compartment A when compartment B no longer exists, this theory caused some consternation — for classical cell biologists, reverse traffic of proteins from the Golgi stack to the ER was a gross heresy.

Lippincott-Schwartz *et al.*² have now gone on to explore the effects of brefeldin in more detail, and have refined their model into a scheme that should excite rather than offend traditionalists. The first series of experiments investigate the features of this retrograde transport by following an early-stage Golgi marker, mannosidase II. The process is inhibited by lack of ATP and slowed at low temperature, which allows intermediates in the process of redistribution to be identified. The mannosidase is initially found in long tubular projections emanating from the Golgi, which indicated that microtubules could be participating in the transport process. Sure enough, the anti-microtubule drug nocodazole prevented the appearance of mannosidase II in the ER induced by brefeldin; however, it did not affect the departure of the protein from the ER after brefeldin had been removed. Mannosidase II is normally found in the early stages of the Golgi complex. The previous report¹ implied

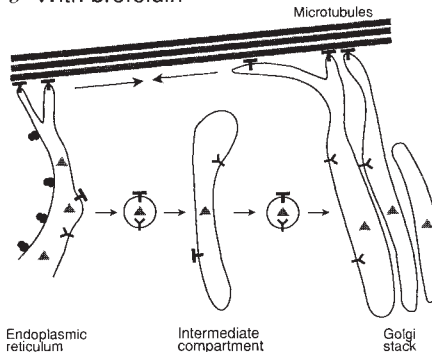
that the late Golgi is not affected by brefeldin, but now the authors observe that another Golgi protein, galactosyltransferase, which is a late-stage marker, is also redistributed to the ER under the influence of brefeldin.

Next the authors turned their attentions to the effects of brefeldin on a compartment proposed to exist between the ER and Golgi, the subject of a previous News and Views¹⁰. Even without morphological evidence, cell biologists would have been obliged to invent this compart-

a Normal



b With brefeldin



A speculative model for the action of brefeldin.

a, Normally proteins are sorted in the intermediate compartment into one class of vesicles containing secretory proteins (triangles) and another containing proteins for recycling to the ER, such as the hypothetical microtubule attachment proteins (T) and the 53K marker protein (Y). The second class can then attach to microtubules and spontaneously fuse with the dynamic ER. *b*, Brefeldin prevents the sorting of proteins from the intermediate compartment, allowing microtubule-attachment proteins into the Golgi, which as a result eventually becomes part of the ER. ment on functional grounds⁹, but now a potential marker for it, a protein of relative molecular mass 53,000 (53K), has been described¹¹. The distribution of this marker was altered by brefeldin, but it did not coincide with the ER. Yet nocodazole and brefeldin in combination caused the 53K protein to co-localize with galactosyltransferase. By contrast, none of these treatments affected a marker for the ER itself.

At this point the authors suggest that the steady-state distribution of the 53K

protein is a balance of anterograde transport from the ER to the intermediate compartment, which is not affected by nocodazole or low temperature, and retrograde transport back, which is blocked by both. Indeed, low temperature alone causes the 53K protein to concentrate in a Golgi-like region, but again with tubular extensions emanating from it. Raising the temperature brings the protein transiently to the ER, before re-establishing its original distribution. Finally, brefeldin and low temperature together also cause the 53K protein and galactosyltransferase to co-localize, but this time in a Golgi-like structure, again with tubular elements.

The authors rationalize their results in an elegant and largely non-heretical scheme. First, they note that the ER is not a static structure, but a dynamic network of tubular membranes which are continuously extending along microtubules and breaking and fusing with each other¹²⁻¹⁴. Second, as originally proposed¹⁰, the intermediate compartment is the destination of both secretory and KDEL-type proteins transported from the ER, but after their arrival, the two are separated. The secretory proteins proceed by the classical route, which does not involve microtubules, to the Golgi, whereas the KDEL-type proteins return to the ER¹⁰ in a step that does. The first effect of brefeldin, then, is to cause fusion of the intermediate compartment and the Golgi. Lippincott-Schwartz *et al.* also propose that brefeldin prevents the intermediate compartment from distinguishing between secretory and 'recycling' proteins, thereby allowing all the leaving vesicles to associate with microtubules and return to the ER.

The second postulate is interesting in itself, in that 'unsorted' vesicles would be delivered to the Golgi. So the ER proteins for microtubule attachment would now appear there, causing the Golgi to produce extensions along microtubules (see figure). In other words, brefeldin does not so much induce transport of proteins from the Golgi to the ER as convert the Golgi into part of the ER, because both compartments can now interact with microtubules in the same way. This scheme has several attractions. It simplifies the process of returning vesicles from the intermediate compartment to the ER: rather than requiring any 'targeting' molecules, such vesicles would by definition contain the (so far unidentified) proteins responsible for attaching the ER to microtubules, and hence could spontaneously join in the dynamics of ER formation. These attachment molecules would normally be excluded from secretory vesicles, preventing their appearance in the Golgi. (Golgi stacks probably have a separate mechanism for associating with microtubules, but this does not seem to

deform the stack or cause the formation of tubular membranes¹⁵.) Conceivably, a change in environment between the intermediate compartment and the ER could temporarily block the action of the attachment proteins, just as it could explain the binding of KDEL proteins to a hypothetical receptor in the intermediate compartment but not in the ER¹⁰.

The behaviour of the KDEL proteins prompts some specific predictions. Lippincott-Schwartz *et al.*² have used markers that are fixed in the ER¹⁶ and are unmoved by brefeldin or nocodazole. In contrast, KDEL proteins would be expected to be more sensitive: nocodazole should cause them to accumulate in the intermediate compartment, and brefeldin at low temperature should put them in the Golgi-plus-tubules structure, along with the 53K protein and galactosyltransferase. Another critical test, as the authors point out, would be to verify by electron microscopy that the 53K protein and galactosyltransferase really are in the same Golgi structure after these treatments, rather than just coinciding at the light-microscopic level. Perhaps the most pressing task, considering that brefeldin acts at concentrations down to 10^{-8} M (ref. 2), is to identify its cellular target, surely a fascinating molecule.

The early years of studying the ER and Golgi were spent defining their morphologies and functions, and mapping the secretory route through them. The next stage — identifying the proteins mediating membrane traffic — is well underway. Now it seems that in the third phase we might revert to asking a simpler but more profound type of question: what is the ER and what is the Golgi? Naive responses might be “membranes that contain the proteins necessary for dynamic interaction with microtubules” (for the ER), and “membranes that don’t” (the Golgi). And the intermediate compartment? On this view, the answer is “membranes that contain the proteins but cannot use them”. □

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Simmering stellar surfaces

M.G. Edmunds

THE visible surface of the Sun is not smooth, but shows a mottled or granular structure of bright patches separated by darker intergranular lanes. Because the Sun is a fairly normal star, it is expected that other stars will also show granulation on their surfaces. A series of papers¹ by D. Dravins and Å. Nordlund now makes available some fine work which simulates the dynamics of rather ordinary stellar atmospheres, and describes the effects of the resulting granular structure. As well as giving a picture of stellar surfaces, the calculations go a long way to resolving a long-standing uncertainty in the interpretation of the velocity fields in stellar atmospheres.

The surfaces of virtually all stars, except for the Sun, are too far away for astronomers to resolve significant surface detail. The observational problem is attributable both to the available diameter of telescopes (limiting the spatial resolution by the well known Rayleigh criterion of optics), and the smearing effect of the Earth's atmosphere. Eventually resolution will be improved by the development of optical interferometric arrays, and by putting interferometers in orbit outside the atmosphere, but a detailed direct view of stellar surfaces is still quite a long way in the future. Nevertheless, the expected granular structure of the stellar atmospheric region (which is termed the surface of a star) can be predicted by modelling of the hydrodynamics of its atmosphere, and the consequences of the structure investigated. Dravins and Nordlund's work has been in progress for well over ten years,

and glimpses of it have been available in conference proceedings, but the extended publication at long last will be heartily welcomed by many stellar astronomers.

The basis of the calculations is a three-dimensional time-dependent hydrodynamic simulation of the gas motions at and just below the visible surface of model stars. As well as the convective motions which transport heat up from the interior, the computer model includes treatment of the transfer of radiation in both the continuum and spectral lines. The importance of including the spectral absorption lines is that their observed profiles, although inevitably summed over the whole disk of a distant star, provide diagnostics of the surface granulation even when the spatial structure of the disk cannot be resolved.

The calculations were performed on a $32 \times 32 \times 32$ grid, with periodic boundary conditions on the two-dimensional planes representing successively deeper layers of the stellar atmospheres. Consuming large amounts of CRAY-1 supercomputer time in the effort, the authors found a characteristic structure for the convective motions. Large, warm, slowly rising granules form and are separated by much narrower regions where ‘fingers’ of cooler (and hence darker) material falls rapidly back to lower layers to be subsequently recycled. The bright rising granules can be seen to grow and then split up. The narrow, fast, cool intergranular lanes probably provide a good channel for the concentration of magnetic fields — and although magnetic fields were not included in the present calculations, it may reasonably be expected that their presence in these regions will provide a suitable route for the transfer of energy by magnetic waves to heat the chromospheric and coronal regions above the photospheric surface.

This sort of model is very familiar to solar physicists, and the interest lies in the extension to stars similar to the Sun, but at least a bit different in temperature and surface gravity. A modest increase in the average granule size occurs as the gravity is lowered. Unfortunately the calculations have not yet been extended to the really low gravities of red giant and supergiant stars, which would show whether just a few (perhaps observable) huge granules result, as has sometimes been conjectured². An apparent complication is that the granules may be easier to see as concentrated regions in stars rather hotter than the Sun, whereas in cooler stars the convective elements — which form the granules — may

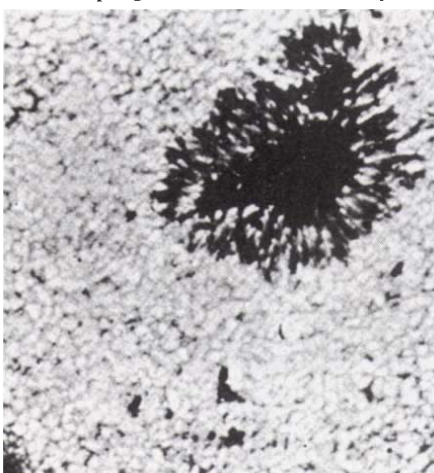


FIG. 1 Solar granulation — a photo of a small section of the Sun with very high spatial resolution. The dark umbra of a sunspot can be seen and small dark pores. The solar surface is covered by a pattern of bright so-called ‘granules’ separated by a dark intergranular network. (From Unsöld 1982; photo by Schwarzschild.)

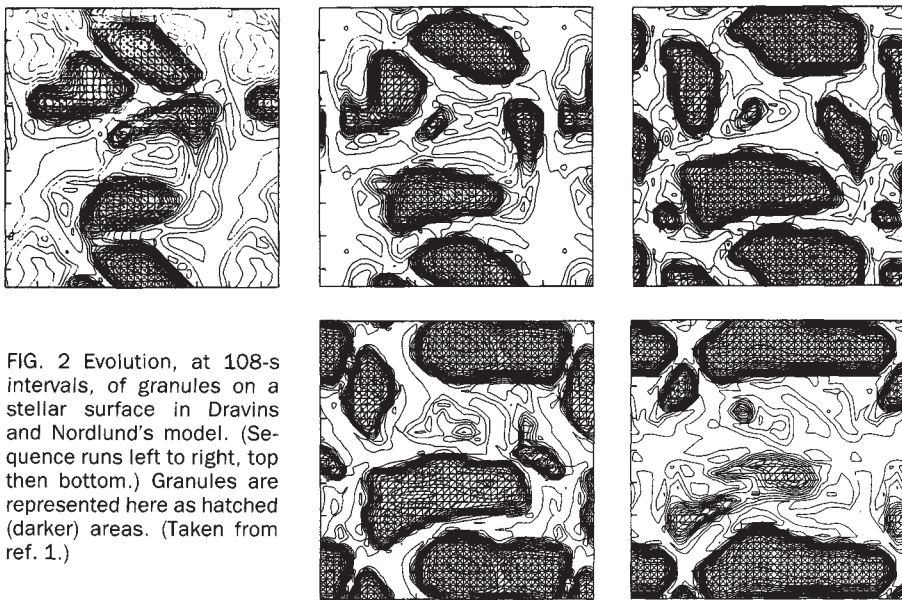


FIG. 2 Evolution, at 108-s intervals, of granules on a stellar surface in Dravins and Nordlund's model. (Sequence runs left to right, top then bottom.) Granules are represented here as hatched (darker) areas. (Taken from ref. 1.)

stop well below the visible surface.

Looked at from a long way off, the stellar surface presents radiation from a whole spread of velocities, temperatures and pressures. This is in very marked contrast to the simple, two-dimensionally homogeneous, stellar-atmosphere models that are typically used in the analysis of stellar spectra. It would be impossible to analyse every stellar spectrum using the complexities of these new simulations, but the calculations are very important in showing up the limitations and strengths of the simplified models. An important result is that the spectral absorption lines show broadening and profiles that are quite typical of observations. For nearly 50 years, controversy has raged about the reality or otherwise of hypothetical turbulent velocity fields in stellar atmospheres which are invoked³ to explain the observed shapes and widths of spectral lines. These turbulent velocity fields, particularly the so-called 'micro-turbulence', were strongly attacked as unphysical in the pages of *Nature*⁴ in the early 1970s. But, lacking anything better, stellar analysts have continued to use them. What the new calculations show is that the combination of temperature and pressure inhomogeneities, and convective mass motions, is sufficient to provide the line broadening (in most cases) without having to introduce further turbulent velocity fields.

It remains to be proved that reasonable abundances of chemical elements can still be deduced by using the convenience of introducing a microturbulence broadening parameter if required for strong lines, and that the weaker lines (whose strengths are less affected by velocity fields) can be analysed with a mean vertical atmospheric structure of temperature and pressure. At least there is now a reasonable physical model of which such questions can be asked. The success that

Dravins and Nordlund have had in their detailed modelling of lines from a few stars using abundances and mean atmospheric physical conditions deduced from classical analyses argues that classical analysts may (when not observing) sleep a little easier at nights.

Some observational confirmation of the general features of the stellar granulation models comes from careful study of the asymmetry of spectral line profiles — in particular the shape of the locus formed by the mid points between the positions of equal intensity down either side of an absorption-line profile. These line bisectors have shapes that are influenced by the horizontal spatial inhomogeneity of the stellar surface. Dravins⁵ has already been able to make some estimate of the relative areas of surface covered by rising granules in stars of different temperature.

The current computational models cannot be regarded as complete because, as well as not including magnetic fields, the calculations assume that the fluids involved are incompressible. This eliminates any effects due to wave motions. As the authors admit, wave motions may have some part to play both in energy transport and in line broadening. But to make really significant further progress it is estimated that a further two orders of magnitude in computing power is needed. It would be a rash man who claimed this will not have happened before another ten years have passed. □

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Nervous tapping

ANIMAL nerve fibres are extremely well insulated from each other, and impulses travelling along them create almost no external field. This minimizes their mutual interference and cross-talk, but hampers those neurologists who want to eavesdrop on their traffic. Now Daedalus is coming to the rescue. He points out that the ion flow accompanying a nerve impulse alters the volume of the nerve slightly, and therefore launches an ultrasonic wave into the surrounding tissue. A modern sensitive piezoelectric transducer should be able to detect this wave at the skin surface. Indeed, a pattern of such transducers, registering the time of arrival of the ultrasonic wave at various points on the skin, could even locate the origin and direction of the wave.

So Daedalus is inventing neuroseismology. DREADCO's engineers are building piezoelectric armbands, anklets, necklaces, and so on, designed to read the nerve traffic in the arms, legs and necks of volunteers. The ultrasonic disturbance accompanying a moving nerve impulse will race out ahead of it and behind it, producing at the armband a complex 'signature' encoding the position, velocity and direction of the impulse. Modern nanosecond-resolution electronics and high-speed data processing will be stretched to the limit to disentangle the superimposed signatures of many close-packed nerves all firing rapidly, and to identify them by location and transmission velocity; but the thing seems feasible. Once the pilot rig is working, the DREADCO team will ask their volunteers to bend each finger in turn, for example, or prick their hands in various places, so as to build up a 'map' of which nerves do what in the arm.

When perfected, the neuroseismograph will be a wonderful diagnostic tool. All those mysterious aches and pains that are so hard to pin down will at last be located and scrutinized. The nerve traffic sustaining muscular tics and spasms, phantom sensations and the like, will also be immediately apparent. A sequence of neuroseismograms made over time would give a marvellously detailed insight into the progress of treatment of such disabilities.

But Daedalus reckons his neuroseismograph will be most useful as a biofeedback monitor. All sorts of skills, like those of the golfer, musician, and typist, depend on establishing proper reflexes in the arms. A trainee wired to a neuroseismograph could immediately compare his nerve recordings with those of an expert and see where he was going wrong. He could polish his skills until the right muscles were receiving the most effective pattern of reflex commands for the job. Even the trickiest skill of all, complete muscular relaxation, could at last be easily assessed and improved.

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Pyrite and the origin of life

SIR—R. J. P. Williams in his recent *News and Views* article¹ raises among other problems the following: "... we must search for the functional advantage for the material [such as FeS₂] in the biological niche in which it is synthesized". A detailed hypothetical answer to this problem, as far as pyrite is concerned, is central to the recent theories of the origin of life due to G. Wächtershäuser. The answer is formulated in the title of the first of his papers on the origin of life: "Pyrite Formation, the First Energy Source for Life: a Hypothesis"².

In this paper, Wächtershäuser notes that certain anaerobic bacteria combine archaic features with a chemoautotrophic metabolism. Starting from this observation, he recommends "the re-examination of the long neglected view that the origin of life and the origin of [chemo-]autotrophic metabolisms coincide" — in contrast with the widely adopted heterotrophic soup theory, and also with the view that the original metabolism is photoautotrophic.

Wächtershäuser suggests that the neglect of the chemoautotrophic option results from the problem of finding a likely energy source. He proposes the anaerobic biomineralization of pyrite as the first energy source for life, calculating the yield of free energy from this process. (More recently, upon a suggestion by Ian R. Kaplan, he has also calculated the still more highly exergonic formation of pyrite when Fe₃S₄ intervenes.)

This hypothesis suggested further hypotheses of great explanatory and predictive power covering precellular life and the formation of cells. These hypotheses enabled Wächtershäuser to predict that the production of pyrite via carbon fixation (in competition with hydrogen formation) by some of the extant archaic bacteria is highly probable^{2,6}. Possible corroborations of this prediction seem to be offered by the two recent letters to *Nature*^{3,4} discussed by Williams¹.

Another prediction of central importance to Wächtershäuser's theory is the evolution of precellular forms of life (surface metabolists); that is, of monomolecular layers of autocatalytic processes of carbon fixation, bound, *in statu nascendi*, to positively charged mineral surfaces such as pyrite crystals that are biomineralized by the surface metabolists themselves^{2,5,6}. This prediction shows a striking similarity with Williams's suggestions that the "surfaces of iron sulphide minerals could also have been catalysts [for the metabolism]".

Indeed, Wächtershäuser points out that his surface metabolists act as catalysts for the biomineralization of pyrite, whereas the pyrite surfaces act as catalysts for the

autocatalytic surface metabolism^{2,6}. Another of his predictions (also possibly supported by Farina *et al.*³ and Mann *et al.*⁴) implies that the surfaces of biogenic pyrite are binding, *in statu nascendi*, membranes of lipids and of anionic peptides⁵, a hypothesis which could explain the otherwise still unexplained asymmetry in the structure of the membranes.

These hints may, I hope, draw attention to a new theory — to my knowledge, the first radical alternative to Haldane's and Oparin's soup theory. A workable alternative seems to me urgently needed, especially in view of the low temperature of the soup required by the most prominent representatives of the theory⁷. This temperature requirement seems to me contradicted by the logic of the geophysical situation as well as by the empirical findings of Woese: both suggest a hot origin of life (with which Wächtershäuser's theory is compatible). Incidentally, Woese's hot origin hypothesis appears to render sterile all those speculations about hot-temperature sterilization, due perhaps to the impact of meteorites.

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SIR—Wächtershäuser^{1,2} has described how the exergonic formation of pyrite (FeS₂) could have been the electron-donating energy source for an autotrophic origin of life. Early Archaean submarine hot springs of moderate (100–200 °C) temperature may have issued through fine iron monosulphide tubes comprising natural chemical gardens — catalytic flow reactors in which the first metabolists could have been cultured³. Hall *et al.*⁴ have also argued for an ancient origin of ferredoxins, the iron-sulphur-proteins that occur in all types of organisms, which are involved in a wide range of biochemical reactions as agents for electron transfer, but which originally catalysed anaerobic fermentation⁵.

We suggest one more important role for iron sulphide, that of nucleating the membrane of the earliest cell walls. We have demonstrated that iron monosulphide gels can form macroscopic spherical shells 1 to 20 mm across³. On a microscopic scale, spherical aggregates of pyrite about 5 µm in diameter are found within fossil hydrothermal chimneys. These framboids appear to have grown inorganically from a

spherical shell of iron sulphide gel. The pyrite crystallites comprising the interior of these framboids are about 0.1 µm across, and so are similar in size to those found in the magnetotactic bacteria.

In a hydrothermal chimney, the iron sulphide shells would initially have consisted of periodic arrays of iron monosulphide crystallites, which would have grown by the diffusion of iron from an aqueous phase into polysulphide droplets. These shells would have provided a site for concentration of polar organic species, by adsorption onto the iron sulphide surfaces.

Once a critical concentration of polar organics had been adsorbed, further growth of the iron sulphides would have been inhibited. Then the organics could become inherently ordered to a liquid crystalline structure. The periodic arrays of the iron sulphide crystallites may have assisted this ordering, developing an organic layer with varied degrees of structure similar to those reported by McConnell *et al.*⁵ in phospholipid membranes. A further concentration of organics onto the first adsorbed layer could follow, the whole comprising a cell membrane precursor.

Similarities between the iron sulphide bearing bacteria and the earliest stages of framboidal iron sulphides give further support for the view that inorganic and organic evolution to the earliest life forms relied on a redox reaction involving carbon fixation concomitant with the oxidation of iron monosulphide to disulphide.

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Geomorphology and mantle plumes

SIR—The recent article by Cox¹ is valuable in highlighting the potential significance of geomorphic data in assessing models of continental rifting and associated surface uplift. In his analysis of the geomorphic evidence for the plume-related surface uplift model proposed by White and McKenzie², there are, however, important elements that permit well supported alternative interpretations.

Here I focus on the geomorphic evidence for plume-related uplift in Africa and on the more general question of the use of drainage patterns in the reconstruction of surface uplift histories.

Cox argues that drainage patterns indicative of topographic doming can be preserved for up to 200 Myr, and that they can therefore provide a basis for testing uplift models. Although he acknowledges cases in which initial drainage systems have been modified significantly, Cox's key point is that rifted domal uplifts create half-radial drainage patterns which can be preserved sufficiently to allow the identification of gross palaeotopography. The development of such drainage patterns in response to rifting has been pointed out previously^{3,4} but, because the primary fluvial systems formed consist of consequent (dip) streams, the resulting half-radial drainage patterns are essentially a reflection of gross topography. It is topography, therefore, that should be the focus of investigation, with fluvial systems being analysed where drainage patterns are anomalous.

One of the proposed plume-related uplifts considered by Cox is the Cape Angola high. It is clear that, even on a broad scale, the morphology of this region consists of two, rather than one, major areas of elevated topography (the Bie Plateau in the north, and the high terrain in central Namibia culminating in the Awas Mountains in the south) which are separated by the Etosha Basin, where the elevation falls to below 1,200 m. Drainage patterns reflect this topography, although Cox points to the anomalous course of the westward-draining Orange River across the extreme southern flank of this hypothesized zone of plume-related uplift. This river course, and the incision of the Orange below the Augrabies Falls, is interpreted by Cox as evidence of drainage antecedence, but there is a significant body of literature extending back to early this century (see ref. 5 for review) demonstrating — on the basis of geomorphic, stratigraphic, sedimentological, palaeontological and zoological evidence — that the palaeo-Orange has shifted its outlet significantly since the formation of the South Atlantic. Capture of dome-flank drainage by rift-related river systems can account for the present-day incision of the lower Orange, as also can concurrent local surface uplift resulting from a flexural

isostatic response to denudational unloading focused in the vicinity of the Great Escarpment. Cox's discussion of the river patterns in the vicinity of the lower Zambezi similarly ignores well substantiated major changes in the drainage of this area during the Late Cenozoic⁶.

Cox also attempts to reconcile the single uplift event suggested by the plume model with what he interprets as firm evidence of episodic uplift and denudation in the form of cyclic erosion surfaces. Such reconciliation may, however, be unnecessary, because the interpretation of the landscapes of southern Africa (and other Gondwana terrains) in terms of episodic surface uplift and denudation has been strongly challenged^{7,8}, both on the basis of the techniques used to correlate erosion surfaces and in terms of the evident structural control of many such surfaces. Moreover, fission-track studies have indicated significant depths of post-rifting erosion extending far inland along a

number of passive margins, including southwest Africa, and these cast doubt on existing denudation chronologies based on erosion surface correlation. Such thermochronological investigations arguably provide the most promising basis for unravelling the key problem of the morphological evolution of passive margins.

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Wasps fail to make distinctions

SIR—A recent experiment¹ showed that worker honeybees can discriminate degrees of relatedness among their female colony mates and that they preferentially aid closer relatives. If this ability were general, our view of the organization of colonies of social insects would have to be radically revised. Instead of working together for the reproductive good of the colony, workers could each pursue partially independent reproductive strategies, each trying to aid its closest relatives. But we have data indicating that females of the paper wasp, *Polistes annularis*, either do not possess this ability or do not use it effectively.

In the spring of 1988, we collected recently-founded colonies of *P. annularis* at a site at which we had marked individuals with colony-specific two-colour paint marks the preceding autumn. Of those individuals that retained both colour marks in the spring, 98% of colony-mate dyads shared the same colour combination, confirming the previously known fact² that spring associations consist of females reared on the same natal nest the previous autumn. But when at least two spring nests arise from the same autumn nest, females are presented with a choice: do they then discriminate degrees of relatedness and prefer to join a subset of natal nestmates to whom they are most closely related?

We have studied females given a clear opportunity to choose among at least two sets of natal nestmates from 29 fall colonies that gave rise to at least two nearby spring colonies. (Those further than 5 m from their natal nest were excluded.) We estimated the relatedness³ of the 222 females retaining both of their identifying marks by starch gel electrophoresis of

proteins. What we find is that the average relatedness to spring colony-mates ($r_1 = 0.469$) is indistinguishable from that to other females from the same autumn nest ($r_2 = 0.452$), suggesting that no preference is expressed. Of course, some degree of discrimination could be missed because of sampling error, but the 95% confidence interval for the difference $r_1 - r_2$ runs from -0.066 to $+0.099$, showing that we can exclude any discrimination larger than 0.1.

Either *P. annularis* females lack the ability to perform such discrimination, or they fail to use it effectively in a natural situation where it would be highly advantageous. The choice has crucial consequences for inclusive fitness because most females (74%) end up being subordinates who reproduce indirectly by aiding the dominant egg-layer². Since average relatedness among fall nestmates is rather low, aiding randomly chosen natal nestmates would make a small contribution to inclusive fitness. At least some super-sisters ($r = 3/4$) are likely to be available, and preferentially helping them would be a far more effective genetic strategy.

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Corrections

■ The title of the Scientific Correspondence, P. A. Kitchin *et al.* *Nature* **344**, 201 (1990) should read: "Avoidance of PCR false positives".

■ Line 67 of M. Super *et al.* *Nature* **344**, 113 (1990) should read: "With CF, 100,000 pregnancies would require 104 amniocenteses which would detect 26 of 40 cases (arithmetic available on request)."

Tubulin site interpretation

SIR—Paschal *et al.*¹ conclude that an amino-acid sequence at the carboxy terminus of α - and β -tubulin is a common

influence both tubulin assembly and the binding of MAP2 and dynein, raising the possibility that some MAPs interact

SEQUENCES AND OCCURRENCE OF β -TUBULIN.

Class	Sequence from residue 422	Brain	Red blood cells
I	yqyyqdataEEEE-FGEAAKEEA	3	10
II	yqyyqdataDEQGE-FEEEGEEDA	58	0
III	yqyyqdataEEEGEMYEDDEESEQGAKE	25	0
IV	yqyyqdataEEEGE-FEEAAKEEA	13	10
V	yqyyqEataNDGEEAFEDDEEINE	0	0
VI	yqyyqdataDVEE--YEEAEASPEKET	0	80

Isotype classes are shown to the left of the amino acid sequences. The last two columns show the percentages of total brain and erythrocyte tubulin, represented by β , of the classes shown (our unpublished observations).

site of interaction for the microtubule-associated proteins (MAPs) cytoplasmic dynein and MAP2. The identity of the site was inferred from the failure of tubulin subunits, cleaved with proteases near their carboxy termini at sites mapped by specific tubulin antibodies, to interact productively with dynein or MAP2. The conclusion is appealing because it suggests a common, perhaps universal, MAP-binding motif E(G/A)EE.

But we believe this interpretation requires fuller consideration. Although the authors note that the same binding domain is found on known α - and β -tubulins from various species, a comparison of the subunits that would compete for MAP binding within a single organism, or even within the same cell, is also relevant. The table shows the carboxy-terminal sequences of all the β -tubulins in the chicken². Remarkably, the supposed MAP-binding domain does not lie in a domain conserved among all β -isotypes, but in the hypervariable region that distinguishes the isotype classes from each other³. Only three of the six subunits carry the supposed binding sequence which, indeed, is missing in Class III β -tubulin expressed, like MAP2, exclusively in neurons.

It is also relevant that there are several high-affinity binding sites ($K_d = 5 \mu\text{M}$) on MAP2 (and also on tau) for a peptide containing residues 422–434, (ref. 4) which is almost invariant among known β -tubulin.

Finally, one of us (D.B.M.) has shown that MAP2 binds nearly equivalently (and with high affinity, $K_d = 0.1 \mu\text{M}$) to microtubules composed either of brain or erythrocyte tubulin, even though the class VI β -tubulin mostly involved does not contain the Paschal sequence and is very different from the other isotypes.

Thus the primary site of MAP interaction probably lies not where Paschal *et al.*¹ suggest, but in the neighbouring conserved region of β -tubulin molecules. Yet the acidic variable carboxy termini clearly

account for continuing dynein-based motility even in neurons in which MAP2 is abundant.

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PASCHAL *ET AL.* REPLY — Murphy *et al.*, in discussing the importance of the tubulin sequence identified in our study, fail to address the role of α -tubulin in the interaction with dynein and MAP2, and ignore evidence contradicting a role for the more highly conserved β -tubulin region.

Our results¹ addressed α -tubulin most directly. In chicken blood-forming tissue and circulating erythrocytes the predominant α -tubulin isoform is α -1, which, in fact, contains the sequence EGEE^{5,6}. About 20 per cent of chicken erythrocyte β -tubulin also contains the related sequence EAEE (see table). In view of the well-known substoichiometric binding of MAPs to microtubules at saturating MAP concentrations, there is a substantial level of α - and β -tubulin isoforms in the erythrocyte containing our proposed binding sequence. We also note that the chief β -tubulin in the erythrocyte contains the sequence EEAEA, which could be functionally related to our proposed EEAE.

With respect to earlier evidence on the MAP2–tubulin interaction, Maccioni

preferentially with specific tubulin isotypes. Selective binding to β tubulin isotypes specific to the brain could be significant in relation to the observation of Paschal *et al.* that MAP2 blocks the interaction of dynein with tubulin in the brain: preferential MAP binding to particular isotypes could

*et al.*⁴ reported on the affinity of the β -tubulin peptide 422–434 but made no direct test of the extreme carboxy-terminal region. Littauer *et al.*⁷ found MAP2 binding to peptides from several regions in α - and β -tubulin but, significantly, no binding to the highly conserved β -tubulin sequence consisting of amino acids 416–431.

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Sex, growth and chance

SIR—In his News and Views article¹, Paul Burgoyne suggested that the *ZFY* and *ZFX* zinc-finger genes on the human sex chromosomes may speed up the development of somatic cells as a prerequisite for the differentiation of the testis. Lack of *ZFY* could therefore lead to failure of masculinization in XX patients presumed to carry the testis-determining *TDY* gene. Phenotypically, such patients can be male, female or hermaphrodite, but neither of the explanations discussed by Burgoyne to account for this variability — X inactivation and functional interchangeability of *ZFY* and *ZFX* gene products with quantitatively different effects — is supported by evidence from human cytogenetics.

When trying to explain the genetics of sex reversal, we need to abandon the search for models which have been successful in unravelling the basis of enzyme defects and confront the problem of cells growing at different rates, where chance may have the last word². When I first suggested that sex differentiation is initiated by differential growth³, I also suggested that threshold dichotomy⁴, or quasicontinuous variation⁵ provided the underlying mechanism. Here, a genetic system resulting in an apparently qualitative difference is caused by quantitative variation with a threshold effect, giving rise to two (almost) distinct classes. The concept was originally invoked to explain the basis of characters like extra toes in guinea pigs⁴ and missing teeth in mice⁵, which have an obvious genetic basis but are not transmitted according to mendelian rules. Neural-tube defects in mice can be caused by incompatible growth rates between different interacting tissues⁶, providing further support for the idea that the genetic basis of sex reversal is similar to that of spina bifida, which also has a genetic basis without a clear-cut pattern of inheritance.

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The Y chromosome functions to minimize the chance element in XY individuals and to maximize the probability of their developing as males⁷. Burgoyne suggests this function in humans is carried out by *ZFY*, whereas *TDY* produces a hypothetical substance to induce fetal Sertoli cells. In accordance with Occam's razor, I believe that all the Y-chromosomal DNA sequences concerned with 'testis determination' act by enhancing the growth of the relevant gonadal cells, thus making the probability of testis development a near certainty.

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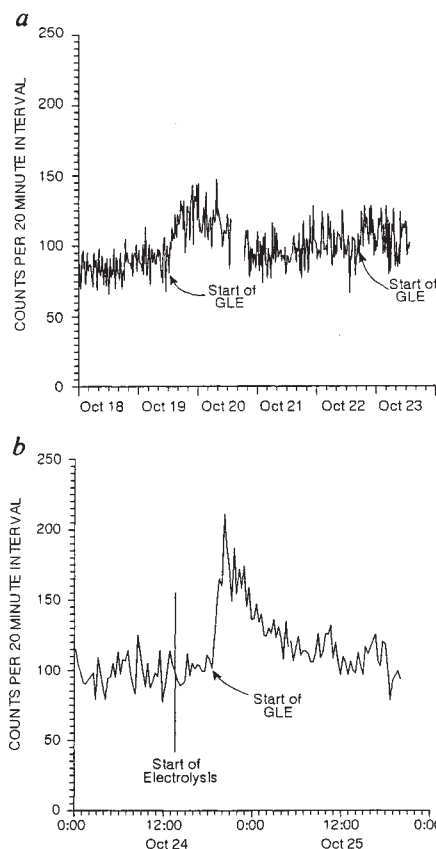
Solar flares and 'cold fusion'

SIR—The production of small quantities of neutrons is one of the characteristics used^{1,2} to attribute the anomalous behaviour of deuterated palladium to 'cold fusion' reactions. In most experiments the neutron signal is barely above the background, and experimental precautions to exclude external sources of noise, followed by careful statistical tests of the data, are essential. Moreover, the fusion signal itself might be quite erratic (R. C. Millikan, personal communication). Distinguishing 'real' bursts of neutron counts from detector noise in such circumstances requires a detailed knowledge of the background. In this note we present neutron counting data showing variations that could easily be attributed to cold fusion, but happen to be synchronized with the terrestrial effects of solar flares. Although cosmic rays have been suggested^{3,4} as possible contributors to the apparent signal of cold-fusion experiments, to our knowledge this is the first time the effect of solar flares has been demonstrated.

Our apparatus⁵ was designed to resemble that of Fleischmann *et al.*¹. The electrolysis cell was located in a dewar at the centre of the neutron detector assembly, which consisted of a cube of polypropylene about 40 cm on a side. Six ³He detector tubes (three each LND Inc. and Texas Nuclear *Textium*; all 2.5 cm in diameter by 30 cm long) were inserted into vertical holes in the block, equidistant from the cell. Sheets of cadmium partially surrounding the detector block absorbed

thermal neutrons, partially reducing the background. Pulse-height thresholds were set to approximately two-thirds of the maximum observed in a spectrum accumulated from a ²⁵²Cf source. The detector efficiency for this calibrated source was just under 5%. The background count rate was 4.5 to 5.0 counts per minute, and data were recorded at 20-min intervals.

A plot of neutron counts against time is shown in the figure (part a). These measurements were taken over the period



Neutron detector reading for the periods: a, 18–23 October 1989; and b, 24–25 October 1989 (Universal Time).

18–23 October 1989 with the electrolysis cell installed but no current applied. Each data point represents the total number of counts per 20-min period. Gaps in the plot correspond to time periods during which the detector assembly was inoperative. A similar plot is shown in b of the figure for the period beginning the morning of 24 October. Current to the cell had been switched on that morning, at 1330 UT (Universal Time).

Both plots show a rise in the count level over a period of several hours. In the figure, b shows a steeper rise, to twice the background level, with a more gradual return to background. We had tentatively attributed this change in signal to fusion events in the cell beginning 5 hours after the start of electrolysis, and gradually coming to a halt over the succeeding 15-h period. The much smaller anomaly in the

background data (a in figure) concerned us, however, so we investigated cosmic-ray and solar activities for a possible explanation.

A compilation of data from the National Research Council of Canada shows that three large solar flares on 19, 22 and 24 October produced high-energy protons that, through interactions with atmospheric nuclei, resulted in increased neutron readings by several ground-based cosmic-ray detectors. These increases are called ground-level events (GLEs). The GLEs occurred at the following times, diminishing to background levels within ~24 hours:

- 19 October: started at 1300 UT; peaked at 1450 UT (40% above background);
- 22 October: started at 1800 UT; peaked at 1900 UT (15% above background);
- 24 October: started at 1825 UT; peaked at 2000 UT (75% above background).

The rises in neutron counts in our detector began on 19 October at 1300 UT (a in figure) and on 24 October at 1900 UT (b in figure). The smaller event of 22 October was barely evident above the background fluctuations of our instrument. Because our anomalies agree in time, relative intensity and duration with the GLEs listed above, we conclude that they were caused by the flares, and not by any reactions in the cell.

Although our experiments have not yet produced unequivocal evidence of 'cold fusion', we note that the neutron detection system is sensitive enough to track solar activity ('hot fusion'). We have reviewed our data over the entire experimental period beginning in April 1989 in the light of this realization, and urge other researchers to do the same. (Other GLEs occurred on 16 August 1989 at 0100 UT and 29 September at 1300 UT; our detector was not operating at those times.) An understanding of 'cold fusion' phenomena can only be facilitated by free exchange of data from experiments producing negative as well as positive results, and much can be learned from 'false positives' in particular.

Note added in proof: No further GLEs have been detected through to 12 March 1990.

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Down to earth explanations

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The Concise Oxford Dictionary of Earth Sciences. Edited by Ailsa Allaby and Michael Allaby. Oxford University Press: 1990. Pp. 410. £20, \$45.

REVIEWING a dictionary is something of a challenge. The oft-quoted lack of plot prevents the reviewer from commending the lucid development of an argument or, more usually, totally disagreeing with the author's conclusions. Reviewers are advised, among other things, to "convey the scope and depth of the book itself", and that is what I shall try to do.

The scope is outlined in the short preface. The authors define the terms geology, geoscience (incorrect) and Earth sciences, in which they include terms from economic and engineering geology, geochemistry, geochronology, geomorphology, geophysics, mineralogy, palaeoecology, palaeogeography, palaeontology, pedology, petrology, sedimentology, stratigraphy, structural geology, tectonics and volcanology. They also include the philosophy and history of the Earth sciences, including brief biographical notes on important figures, planetary geology, hydrology, oceanography and even climatology, palaeoclimatology and meteorology. All this follows in about 8,000 entries on 410 pages.

So there is the dictionary's scope. It is not an encyclopaedia, but a "dictionary proper, aiming only to define terms, as many as possible and in as few words as possible". Entries consist of two or three short sentences, sometimes more when necessary, with extensive cross-referencing, "paying particular attention to those entries that unavoidably require the definition of other 'embedded' technical terms". This is done by asterisks, and by using the words *see* and *compare*. Not all the entries are new: about one-third come from the *Oxford Dictionary of Natural History* (1985) — but they are extensively sorted and reworked.

For whom is the dictionary designed? The authors, aided by 31 contributors and advisers, aim at "serious students and non-professionals with a deep and abiding interest in the areas covered". Information must be comprehensible to non-specialists and easily accessible; they hope that the dictionary is "user-friendly". It includes a useful bibliography, up to 1987, of 270 references which, although not linked to the entries, could lead the curious along some interesting paths.

Now for the difficult bit: how to judge the accuracy and usefulness of the entries and the value of the cross-referencing system? I decided in the first place to choose three sets of terms: those I know well and which everyone would know

something about, like *volcano* and *soil*; those I know something about but which non-geologists probably would not, like *turbidites* and *trans-Saharan seaway*; and terms which only specialists would know (certainly I didn't) like *Ringwood's rule* and the tongue-twisting *Calyptoptomatida*.

Volcano has a five-sentence explanation, including the asterisked words Earth, viscosity, magma and eruptions, and we are invited to *see* central vent and fissure volcanoes and *compare* Hawaiian, Peléean, Plinian, Strombolian, Surtseyan, Vesuvian and Vulcanian eruptions. If we look up magma, this leads us to 12 more asterisked words, and eruptions, to five more, and so it goes on like a chain letter. On the same page as volcano, there are definitions of volcanic bomb, cone, constructs, dome, dust, pile, neck, plains, plug and units, and volcanic-exhalative processes and volcano-tectonic depression — so there is good and accessible coverage of the subject. The same goes for *soil*, where there are 26 adjacent definitions of soil-related terms, and of course separate entries for gleys, chernozems and the like.

In my second category of terms, *turbidites* shunts us straight to turbidity currents; what they are, where they occur, how they are caused and their ideal accumulation in *Bouma cycles. Under *Trans-Saharan seaway*, we learn about its extent, when it was operating, what faunas migrated through it and what

controlled it.

In my third category, a tough explanation of *Ringwood's rule* had me rushing to look up diadochy (*see* ionic substitution, which I did, and was satisfied). *Calyptoptomatida* is a class of marine molluscs from the Palaeozoic and is described in some detail. How ever did the editors select from the millions of known fossils and the parts they are made of like stipes and brachidia? But I'm glad to know there's a *Supersaurus*.

After this short but satisfying consumer test I was so hooked that I went on to pursue other lines of enquiry. Does the dictionary contain recent terms like underplating, duplex, zap pits, *mise-à-la-masse* method, black smokers and (dare I mention it?) the greenhouse effect? Yes, it does. Does it deal with mathematical concepts such as transmission coefficient, Darcy's law, phi scale and Froude number? Yes, with formulae and explanations of the symbols used. Does it amplify abbreviations and acronyms like HDR, SEM, TEM (given as transient electromagnetic method, not transmission electron microscope), FAMOUS, GHOST and BIRPS? It does, in good measure.

How does it deal with those difficult-to-remember stratigraphical systems, series and stages such as Serravallian and Kasimovian? It explains what lies below and what lies above, and sometimes the equivalent division in other parts of the world. There is information on famous Earth scientists such as Brogniart, Niggli, Pettijohn and Tuzo Wilson (how on earth did the editors limit their choice?), and a few tables with, for example, data on the units of geological time and the 17 satellites of Saturn.

This dictionary is, you might say, a mine of information and I believe that it will serve well both students and practitioners

The print, 'Natives of Aru shooting the great bird of paradise' was reproduced from *The Malay Archipelago* by A. R. Wallace. The book is based largely on four field journals that were kept by Alfred Wallace during the eight years he spent in Malaysia and Indonesia. His study provided extensive information on the flora, fauna and peoples of the area. *The Malay Archipelago* was originally published in 1869 and has been reissued by Oxford University Press as a paperback. Price £10.95, \$29.95.



of Earth science as well as the much larger number of researchers who need to understand the terminology of this rapidly expanding field. I marvel at how the editors have compressed so much so clearly into a manageable, well-printed and inexpensive volume, a feat which occurs only once in a blue moon — and even that is defined! □

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A social science

Christopher C. Booth

The Politics of Evolution: Morphology, Medicine, and Reform in Radical London. By Adrian Desmond. *University of Chicago Press: 1990. Pp. 503, £27.95, \$40.25.*

CHARLES Darwin never claimed to have originated either the concept of evolution or even that of natural selection. In the historical account that he gave on the progress of opinion on the origin of species before the first publication of his great work in 1859, he referred to "thirty-four authors who believe in the modification of species, or at least disbelieve in separate acts of creation". He paid generous tribute to the views of the Parisians, Lamarck and Geoffroy Saint-Hilaire, who by the early nineteenth century had concluded that all forms of life tend to progress and that what we call species are various degenerations of the same type.

In 1826, in Edinburgh, Robert Grant, later to become the first professor of comparative anatomy in the newly founded University of London, declared his belief that "species are derived from other species". Darwin, Grant's pupil in Edinburgh, went on to draw attention to the views of W. C. Wells, who by 1818 had clearly recognized the principle of natural selection, though only in the races of man, and to the previously unnoticed work of the radical social reformer, Patrick Matthew, *Naval Timber and Arboculture* (1831) in which precisely the same view of the origin of species was propounded as was set out in the papers published in the *Linnaean Journal* by A. R. Wallace and himself. Subsequent to those presentations, the distinguished professor Richard Owen seemed to claim that he too had promulgated the theory of natural selection before Darwin, who summarily dismissed Owen's controversial writings as immaterial since both Wells and Matthew had preceded them.

The literature of evolution before Darwin has been too little studied by historians, nor has it been fashionable to set evolution in its social and political context. In a remarkable work, *The Politics of*

■ Also recently published, *Collins Reference Dictionary of Environmental Science* by G. Jones, A. Robertson, J. Forbes and G. Hollier, contains over 1,800 entries of terms in use in the environmental sciences, a multidisciplinary field which has evolved over the past two decades. Published by Collins, price £5.95.

■ Published today is the *New Penguin Dictionary of Geography* by A. N. Clark, a revised and updated edition of the original 1985 publication. Line drawings, graphics and maps are well used to explain and clarify terms. Published by Penguin, price £4.99, \$9.95.

Evolution, Adrian Desmond succeeds in drawing together the strands of scientific thought and radical political philosophy, as well as the personalities and ambitions of those in science and medicine whose activities preceded Darwin's publication.

The original philosophical anatomy of Geoffroy and Lamarck, with its anticlerical implications, was imported into Britain by students returning to Edinburgh who sat at the feet of the radical atheist Robert Knox, soon to be destroyed by the Burke and Hare scandal. Moving to the London of the 1820s, they found a city seething with radicals demanding reform. Reform was in no way restricted to political matters. In 1823, stimulated by William Cobbett, the tempestuous Thomas Wakley had founded *The Lancet*, its carefully chosen name implying its function of incising the abscess on the medical body politic. Polite elitist organizations such as the Geological and Zoological Societies were facing radical reform, and even the Royal Society was in ferment. Birkbeck had founded his Mechanics Institutes and his fellow student from Edinburgh, the future Lord Brougham, was engaged in setting up the University of London, whose anticlerical stance was in striking contrast to the deep-rooted religious conservatism of the ancient Anglican universities.

Robert Grant, closely supported by the radical Thomas Wakley, was the first to use the term 'evolution' in its modern sense. He, like the campaigning Wakley, was a fervent opponent of the medical corporations such as the powerful College of Surgeons, riddled as it was with nepotism. Such institutions resisted reform as much as they objected to the new evolutionary concepts associated with Grant and the rough medical *sans-culottes* who attended the private anatomy schools that the college sought to close down. Stirred to action, the college appointed a young hopeful, Richard Owen, to catalogue their collections and he became the traditional scientific standard bearer for the establishment cause. Knighted in later life, Owen crossed swords both with Darwin and the ambitious Thomas Henry Huxley, but Huxley wrote a generous tribute to him as a comparative anatomist and palaeontologist after his death.

Adrian Desmond concludes his account

with a speculation as to why Darwin took so long to publish his conclusions. It was, he suggests, because Darwin — a Whig conservative — had no desire to see his work used by a crowd of unruly radicals to support their disreputable causes. By the 1850s, the crusading zeal of the radicals had largely died down and Darwin's relationship with Grant had cooled. Darwin's caution, however, could equally have been due to his reluctance to offend the Oxbridge establishment to which he belonged. He was in no way surprised that his old Cambridge teacher, the Trinity geologist and divine Adam Sedgwick, received his copy of the *Origin of Species* "with more pain than pleasure". Nevertheless, Adrian Desmond makes an entirely convincing case.

This work is excitingly written, meticulously researched and unique in its politico-scientific analysis. The illustrations are excellent; grave Victorian faces jostle with the most scurrilous of contemporary lampoons. This fascinating account will be of great interest not only to social historians and scholars of evolution but to all who seek to understand the stormy symbiosis that has always existed between science and society. There are lessons here for those involved in that relationship today. □

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Hot to handle

John Knill

Britain's Nuclear Waste: Safety and Siting. By Stan Openshaw, Steve Carver and John Fernie. *Belhaven: 1989. Pp. 207. Hbk £20, \$35; pbk £8.95, \$15.95.*

RADIOACTIVE waste disposal has been for many years possibly the most important environmental concern in the minds of many people. For this reason, at a time when environmental matters are centre stage, it may be thought surprising that this concern has receded, if only temporarily, from the front pages.

Several factors have contributed to this situation in Britain, at least. The decision to investigate Dounreay and Sellafield as possible sites for disposal defines whose back yard, at least for the present, is involved. Matters are unlikely to come to a head again until Nirex, the nuclear-waste disposal body, makes a choice between these two sites, or rejects both and looks elsewhere. The controversy about the role of nuclear power after the privatization of the electricity industry, which ended last year with the retention of nuclear power within the public sector,

tended to raise, rather than help to resolve, questions in the public's mind about nuclear power. And in the United States, the decision by the Department of Energy late last year to restructure the management of the programme of investigations at the spent fuel repository site at Yucca Mountain, leading to a delay of seven years in planned completion, has hardly increased public confidence. Most recently, the Gardner report tends to deflect inquiry into leukaemia clusters away from waste discharges into the environment towards health and safety at the workplace.

Most authorities now recognize that the disposal of wastes by uncontrolled release into the environment — the 'dilute and disperse' philosophy — is unacceptable. Waste must, therefore, be destroyed, stored or disposed of safely; this issue has to be urgently addressed. Radioactive waste cannot be destroyed, although there may be some loss of toxicity over extended periods of time. Such waste can be stored, and indeed this is the *de facto* policy for high- and intermediate-level waste at the moment because of a lack of available disposal routes. Nevertheless, storage must be regarded in the long-term as temporary; it results in radiation exposure to the industry workforce and proposals to construct new storage facilities face strong local opposition. As a consequence it is perhaps not surprising that safe disposal remains the governmentally preferred option.

This highly readable book addresses the problems of radioactive waste and identification of sites for disposal. The two opening chapters provide an overview of radioactive waste, the national-interest factors and public perception of the issues. The origin, nature and composition of wastes are discussed against a background of relevant events during the past 50 years. The next section covers in a balanced and informative manner the development of government policy and the history of Nirex in Britain. Having set the scene, the authors then describe various disposal routes, including sea dumping; the Drigg low-level radioactive waste-disposal site south of Sellafield; the proposed site for intermediate-level waste in the Billingham anhydrite mine; and the four proposed shallow sites for low-level waste disposal in Britain (Bradwell, Elstow, Fulbeck and Killingholme). This chapter ends with a consideration of the arguments against the four low-level sites, which were abandoned in 1987.

One of the main objectives of the authors is to examine the methods more recently adopted by Nirex for the selection of sites for deep waste disposal and to argue the case for the application of computer-based geographical information systems (GIS) as an alternative approach. The guidance provided by the

UK Department of the Environment in site selection is based on performance criteria for the repository rather than a method statement to achieve that objective. As a consequence, Nirex has developed its own methodology, which has been published in outline. A ranking is achieved by an iterative process, starting with about 500 sites and narrowing the search as the sites are reviewed increasingly critically against specific criteria such as population, geology and transport. This procedure is ideally suited to GIS and it is, in retrospect, surprising that Nirex does not seem to have used this technique which could be faster, more economical and more readily reproducible than the essentially manual systems it actually adopted.

The closing chapter reviews a story which has so far only been partially told, arguing the case for extended storage. The authors include open letters to the

Department of the Environment, Nirex and the Radioactive Waste Management Advisory Committee to illustrate their perception of the way forward.

The authors demonstrate their clear grasp of the subject and can rarely be faulted in their considered, balanced treatment of controversial issues. Anyone who wants to learn about the politics and problems of this subject must start here, and the book should be required reading for those involved in radioactive waste management. Perhaps the authors could have performed an even more valuable task by also including open letters to the anti-nuclear environmental organizations and to the general public. Only then could we learn where their preferences actually lie. □

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Stick together

William J. Sutherland

The Ecology of Bird Communities. Two Volumes. By John A. Wiens. *Cambridge University Press: 1989. Volume 1 pp. 539. £50, \$80. Volume 2 pp. 316. £35, \$65.*

COMMUNITY ecology has had an erratic history. Until the middle of this century the field was dominated by botanists

introduced some exciting and elegant mathematical theory and showed how it was possible to collect relevant field data. This led to an outbreak of research in the United States but never seemed to do the same in Britain. By the mid-1970s, the fundamental concepts of community ecology and the evidence for them were attacked from many directions and new interpretations suggested. Wiens has the difficult job of seeing which fragments remain and whether any fit together.

His two-volume work is written "to

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REASONS

Members of the community — the black headed gull.

and consisted largely of descriptions of habitats and debates as to whether communities actually occurred. In the late 1950s and early 1960s, the subject was invigorated by David Lack, who completed field studies of closely related birds, and by Robert MacArthur, who

provide a critical assessment of the current state of affairs in avian community ecology"; Wiens achieves this objective admirably with a critical and careful review of the evidence for the main ideas and a fair analysis of the more contentious issues. In the first volume he considers the

history and methodology of the subject and reviews the studies relating to the main concepts such as the assembly of communities, number of species, niches, guilds, distributions, density compensation, resources and convergence.

Interspecific competition is usually thought to be the prime factor in determining the main patterns of bird communities. In his second volume, Weins reviews the relevant literature and shows that there are significant problems in most studies and few convincing examples. The best evidence, is for insectivorous birds, and Weins devotes a whole chapter to them. These birds, however, tend to defend interspecific territories, and thus competition between them may be atypical.

It is likely that competition has been overemphasized and that predation, parasitism and disturbance all contribute to the observed patterns. Of these, the role of parasites seems the least understood, but as an example of their possible importance, it seems that the distribution of bird species in Hawaii is related more to the susceptibility of each species to introduced malaria than to competition. How many community ecologists would consider examining the responses of their study species to parasites?

It has often been assumed that communities are in equilibrium, but in reality they are probably fairly dynamic. In many studies, specialists suggest that atypical rain, temperature, wind or some atypical biotic event affected the expected patterns. As Weins points out, such 'atypical' events may occur sufficiently regularly to account for typical patterns of community structure.

Weins points out that the study of bird communities may be harder than Lack or MacArthur imagined. It does seem that the more abstract the measure, the less convincing the results. Thus studies of niches, assemblages and diversity all seem rather unconvincing, whereas those based firmly in population biology seem more successful. The studies that are really illuminating are those, such as Galapagos ground finches and *Parus* in Scandinavia, where the natural history is well known and provides a solid base for detailed studies of ecology and genetics. It seems clear from these volumes that ecologists need to know their species and need to study individuals and populations rather than generalized processes affecting communities. □

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Case study

Peter Gill

DNA Technology and Forensic Science. Edited by Jack Ballantyne, George Sensabaugh and Jan Witkowski. Cold Spring Harbor Laboratory Press: 1989. Pp.368. \$95.

A FEW years ago, a Cold Spring Harbor Laboratory Banbury symposium devoted to forensic science would have been unthinkable. The reason for the recent surge of scientific, legal and media interest in this field lies in the power of DNA analysis. Previously, forensic scientists were used to quoting figures of chance association of the order of one in hundreds using conventional grouping methods, but now estimates of one in thousands or millions are common for undegraded samples.

Forensic biology has understandably attracted attention from those in various disciplines — sociologists, statisticians, and population and molecular geneticists, not to mention the legal profession. The organizers of the Banbury conference attempted to bring these different factions together, perhaps to find common ground, but certainly succeeding in showing the controversies surrounding this new science.

Approximately one-third of *DNA Technology and Forensic Science* is devoted to the socio-legal implications of

DNA analysis. There are lobbies both in the United States and the United Kingdom who wish to profile the DNA of all individuals (males?) at birth. At present, this extreme appears unrealistic, but in the short term DNA databases, in which details of individuals are stored, may be feasible. The implications of such databases are explored here by several authors; for example, they could be used to search for associations of hypervariable loci with other characteristics, such as genetic diseases. And because present-day technologies will be out of date in 5–10 years' time, there is a need to store DNA from convicted people in order to compile new databases in the future. Although most authors recognize the social need for a DNA index, safeguards are needed to ensure that any such database or DNA samples are used only for the purpose originally intended.

Paradoxically, the section "Establishment, maintenance and regulation of databases" contains only one paper describing the practical problems of organizing databases between several laboratories. The conclusion here is that complete standardization of systems is needed. The remaining papers in this section include descriptions of national databases already in use by the National Crime Information Center of the United States (vehicles, guns, wanted people and so on) and details of the human gene mapping library. The emphasis placed on sociological implications of DNA database identification is not balanced by a scientific evaluation of

the problems of implementation.

Eric Lander discusses some of the assumptions of population genetics, such as the problems of correctly identifying a population, of sampling error, or obtaining a truly random sample and of the assumption of Hardy–Weinberg equilibrium. The questions of independence and matching criteria are at present crucial issues in the interpretation of DNA profiles. Any cutoff point (such as ± 3 standard deviations) which the specialist uses to decide whether two bands on a gel match is an arbitrary decision that can be circumvented by the application of bayesian statistics. The absence of papers discussing this technique is a significant gap, as the reader is left with the impression that there is no objective solution on the horizon to the problem of band-match criteria.

Quality control and quality assurance are covered by M. Baird. The lively discussion provoked by an autoradiograph showing a band-shift in a semen stain compared with a suspect's control sample highlights the need for well-defined (and measurable) objective criteria for matching DNA profiles.

Alec Jeffreys provides an excellent review of the development of DNA profiling, from multilocus probes to the use of the polymerase chain reaction (PCR) in coamplifying six loci. Problems associated with the use of PCR in forensic science are discussed by R. Higuchi. It is generally agreed here that it is too early to introduce this technology into casework because of potential problems associated with nanolitre contamination, particularly from PCR products; this would be sufficient to give a false result. One option would be to include identifier plasmid primers in each reaction that could subsequently be detected and shown to be correct. Two papers on automated sequencing are included, but again this technology is too preliminary for practical applications.

The question of the introduction of DNA into forensic casework provoked some revealing discussions, often longer than the papers themselves and so providing a flavour of the differences in opinion associated with DNA profiling. The contributions are mainly North American (only two from the United Kingdom), and so the discussions relate predominantly to the requirements of the North American legal system, such as the Frye test.

This volume is wide ranging, and in this respect unique. The field is now moving so quickly that the controversies on independence and band-match criteria highlighted here will quickly become out of date, although socio-legal debates will continue in the same vein. □

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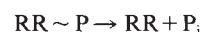
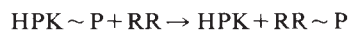
Signal transduction in bacteria

Jeffrey B. Stock, Ann M. Stock & James M. Mottonen

Cells display a remarkable ability to respond to small fluctuations in their surroundings. In simple microbial systems, information from sensory receptors feeds into a circuitry of regulatory proteins that transfer high energy phosphoryl groups from histidine to aspartate side chains. This phosphotransfer network couples environmental signals to an array of response elements that control cell motility and regulate gene expression.

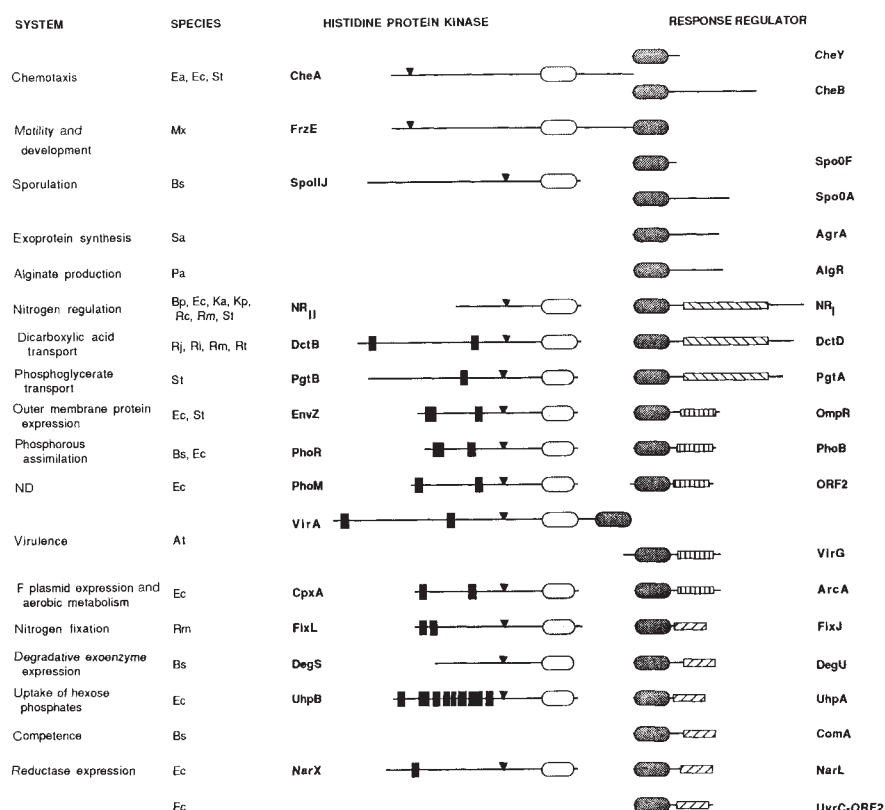
How do cells use information about their surroundings to make appropriate adaptive responses? Studies of the molecular biology of regulatory processes in bacteria have begun to yield some interesting answers. A combination of genetic and biochemical approaches has shown that stimulus-response coupling often involves two families of signal transduction proteins (Fig. 1). One family is defined by a homologous domain that in most, but not all, cases corresponds to the intracellular signalling domain of a membrane receptor protein; the other family is a set of homologous cytoplasmic proteins that function as response regulators, transducing information from the receptors to appropriate adaptive response elements. Members of these two families have been identified in more than 10 different prokaryotic species. Processes regulated in this way include sporulation, transformation competence, pathogenicity, virulence, gliding and flagellar motility, membrane transport and intermediary metabolism. In the 20% of the *Escherichia coli* genome that has been sequenced¹, genes for more than 10 different pairs of these components have already been found. It is conceivable therefore, that in just this one type of cell, as many as 50 pairs of these regulatory elements may exist, each connecting a distinct set of sensory inputs to a specific array of output activities.

In this review we show how the sequence similarities that define each family of bacterial signal transduction proteins can be understood in terms of a common set of enzymatic activities. One component of the regulatory pair is a kinase that uses ATP to phosphorylate itself at a histidine residue. The phosphoryl group from the histidine protein kinase (HPK) is transferred to an aspartic acid side chain within the conserved domain of a second component, the response regulator. The response regulators (RR) control a wide range of cellular activities, including motility and gene expression. The level of response regulator phosphorylation is further controlled by associated phosphatase activities:



This conserved signal transduction mechanism has been directly demonstrated in several systems. The best understood are: (1) regulation of outer membrane porin synthesis in response to changes in medium osmolarity; (2) control of gene expression in response to the intracellular balance between carbon and nitrogen metabolism; and (3) chemotaxis in response

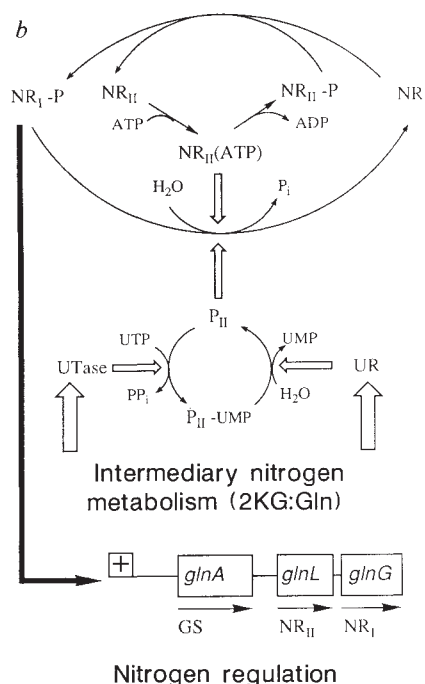
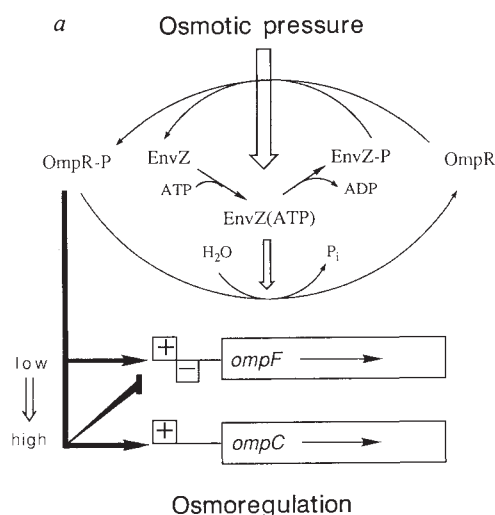
FIG. 1 Two families of homologous signal transduction proteins in bacteria. Histidine protein kinases and response regulators have been identified in a wide range of Gram-positive and Gram-negative species (At, *Agrobacterium tumefaciens*; Bp, *Bradyrhizobium parasponiae*; Bs, *Bacillus subtilis*; Ea, *Enterobacter aerogenes*; Ec, *Escherichia coli*; Ka, *Klebsiella aerogenes*; Kp, *Klebsiella pneumoniae*; Mx, *Myxococcus xanthus*; Pa, *Pseudomonas aeruginosa*; Rc, *Rhodobacter capsulatus*; Rj, *Rhizobium japonicum*; Rl, *Rhizobium leguminosarum*; Rm, *Rhizobium meliloti*; Rt, *Rhizobium trifolii*; Sa, *Staphylococcus aureus*; St, *Salmonella typhimurium*). The kinases share a homologous domain of ~100 amino acids generally located near the C terminus (open ovals). Members of this family have homologous sequences surrounding a conserved histidine residue (inverted triangles), and most members of the family have stretches of hydrophobic residues characteristic of membrane-spanning sequences (filled boxes). The response regulators all share a homologous domain of ~100 residues generally located at the N terminus (filled ovals). Many response regulator proteins fall into one of three subfamilies defined by homologies between their C-terminal domains (hatched and striped boxes). The FrzE and VirA proteins contain both histidine kinase and response regulator domains.



to attractant and repellent gradients. These examples illustrate how the same basic phosphotransfer chemistry can function with disparate auxiliary components to regulate very different processes. Biochemical and genetic results indicate that cross-talk between these parallel signal transduction pathways may provide a mechanism for higher-order information processing functions.

Regulation of porin expression

Bacteria such as *E. coli* have a cytoplasmic compartment surrounded by a periplasmic space. Solutes are actively transported across the inner, cytoplasmic membrane by numerous different permeases, whereas the permeability of the outer membrane is largely determined by a few pore-forming proteins². *E. coli* K12 strains have two main porins, OmpF and OmpC. OmpF pores are slightly larger than those produced by OmpC, and bacteria respond to changes in the osmotic strength of the culture medium with changes in the ratio of OmpF to OmpC. OmpF predominates at low osmotic strength and OmpC at high osmotic strength³.



Porin gene expression is controlled by a relatively simple signal transduction system involving just two proteins: a histidine protein kinase, EnvZ, which transmits environmental information, and a response regulator, OmpR, which controls transcription of the porin genes (Fig. 2a).

EnvZ is typical of membrane receptor proteins with two domains separated by hydrophobic membrane-spanning sequences⁴. The periplasmic domain is presumed to function as

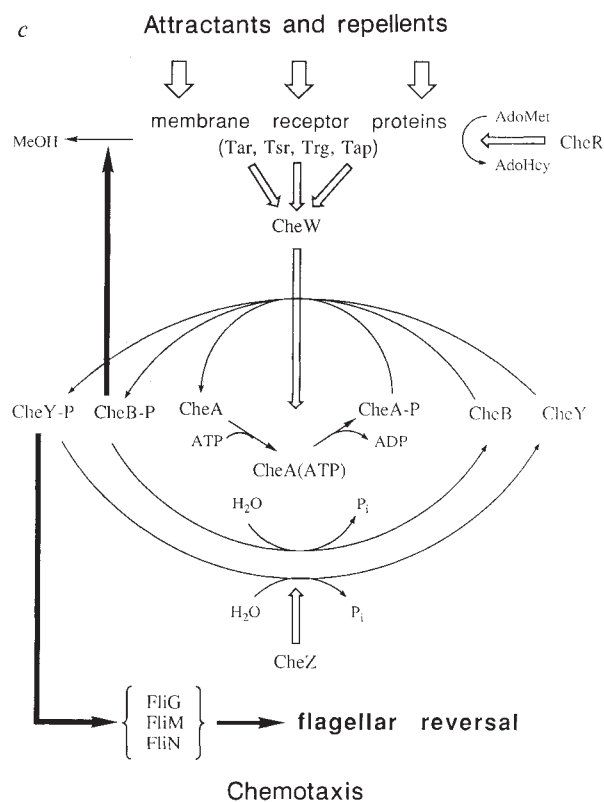


FIG. 2 Signal transduction pathways in bacteria. **a**, In osmoregulation of outer membrane porin expression, a sensory receptor histidine kinase/phosphatase, EnvZ, controls the level of phosphorylation of a response regulator, OmpR. Phosphorylation enhances OmpR binding to DNA sequences upstream of the porin gene promoters. At low levels of phosphorylation (low osmolarity) phospho-OmpR binds to a high affinity site(s) near the *ompF* promoter preferentially activating *ompF* transcription. At higher levels of phosphorylation (high osmolarity) binding to lower affinity sites represses *ompF* and turns on *ompC*. **b**, In the nitrogen (*ntr*) regulon, gene expression is controlled by the balance between carbon and nitrogen metabolism, primarily through the counteracting influences of α -ketoglutarate (2KG) and glutamine (Gln) on the bifunctional uridylyl removing/uridylyltransferase (UR/UT) enzyme that controls the level of uridylylation of the P_{II} protein. High ratios of glutamine: α -ketoglutarate favour UR over UT. The unmodified form of P_{II} activates a phosphatase activity associated with NR_I, the histidine kinase of the Ntr system. This provides a mechanism for regulating the level of phosphorylation of the corresponding response regulator, NR_I. Phospho-NR_I binds to DNA sequences upstream of the promoter for the glutamine synthetase gene, *glnA*, and activates transcription of the *glnALG* operon. **c**, In chemotaxis, a family of membrane receptor/transducer proteins detects attractant and repellent chemicals: Tar is the aspartate receptor; Tsr, that for serine; Trg, that for ribose and galactose; Tap, that for peptides. Receptor sensitivities are regulated by methylation and demethylation at glutamate residues, reactions catalysed by the CheR methyltransferase and the CheB methyltransferase, respectively. The receptors, with CheW, regulate the activity of a histidine kinase, CheA. Phospho-CheA donates phosphoryl groups to two response regulators, CheY and CheB. Phospho-CheY interacts with flagellar motor components (FliG, FliM, and FliN) to induce tumbling behaviour; phospho-CheB is activated to demethylate the receptors. Receptor demethylation attenuates the receptor signal that turns on CheA. The CheZ protein facilitates the hydrolysis of phospho-CheY. The schemes for Omp, Ntr, and Che regulation outlined in **a**, **b**, and **c** are based on studies of these systems in *E. coli* and *S. typhimurium*.

an environmental sensor that controls the signalling activity of the cytoplasmic domain. The cytoplasmic domain of EnvZ is autophosphorylated in the presence of ATP, and this phosphoryl group is then passed to OmpR^{5,6}. EnvZ can also function as a phosphatase to facilitate the dephosphorylation of phospho-OmpR^{6,7}. Phosphorylation enhances the binding of OmpR to the promoter regions of the porin genes⁸. Depending on the particular site involved, binding can either activate or repress transcription⁹.

Regulation of nitrogen metabolism

The central enzyme in bacterial nitrogen metabolism is glutamine synthetase which, under limiting conditions, mediates the entry of ammonia into anabolic metabolism¹⁰. The glutamine synthetase gene, *glnA*, is dramatically induced when cells are starved for nitrogen¹⁰.

The response regulator for the nitrogen (Ntr) regulation

system is a transcriptional activator protein, NR_I, that when phosphorylated binds the *glnA* gene promoter and activates its transcription¹¹. The state of phosphorylation of NR_I is controlled by the histidine kinase NR_{II} whose activation is ultimately controlled by the balance between carbon and nitrogen metabolism as reflected in the relative levels of the intermediary metabolites glutamine and α -ketoglutarate.

Briefly, the ratio of the two metabolites controls the activity of a bifunctional enzyme (UTase/UR) that uridylylates and deuridylylates tyrosine residues in a regulatory protein, P_{II} (refs 10, 12), which in turn controls the activity of the histidine kinase NR_{II} (refs 13, 14) (for more detail see legend to Fig. 2b). Unlike EnvZ, NR_{II} is not a membrane receptor. P_{II} thus acts as the environmental sensor that controls signalling. One advantage of a dissociable regulatory subunit rather than an associated domain, as in the receptor that has this role in the porin system, is its freedom to interact with multiple regulatory components.

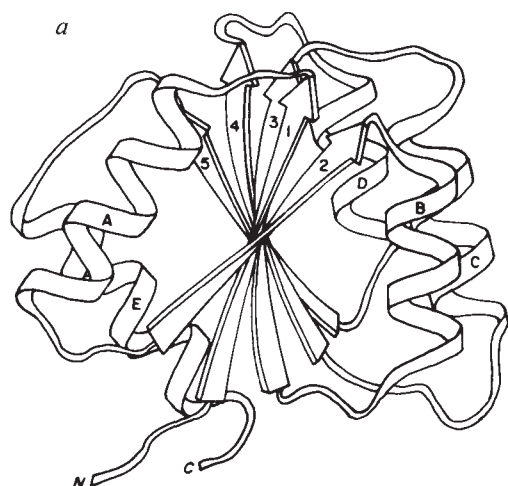


FIG. 3 Relationship of primary structures of response regulator domains to tertiary structure of CheY. a, CheY is a single-domain protein composed of a five-stranded parallel β -sheet (1-5) surrounded by five α -helices (A-E)³⁷. b, Alignments of homologous regions of response regulators with structural elements of CheY indicate a conserved hydrophobic core (shaded residues; see Fig. 4a). The three residues that are highly conserved in the response regulators are boxed. These residues, Asp 13, Asp 57, and Lys 109 are located near the C-terminal edge of the β -sheet (see Fig. 4b).

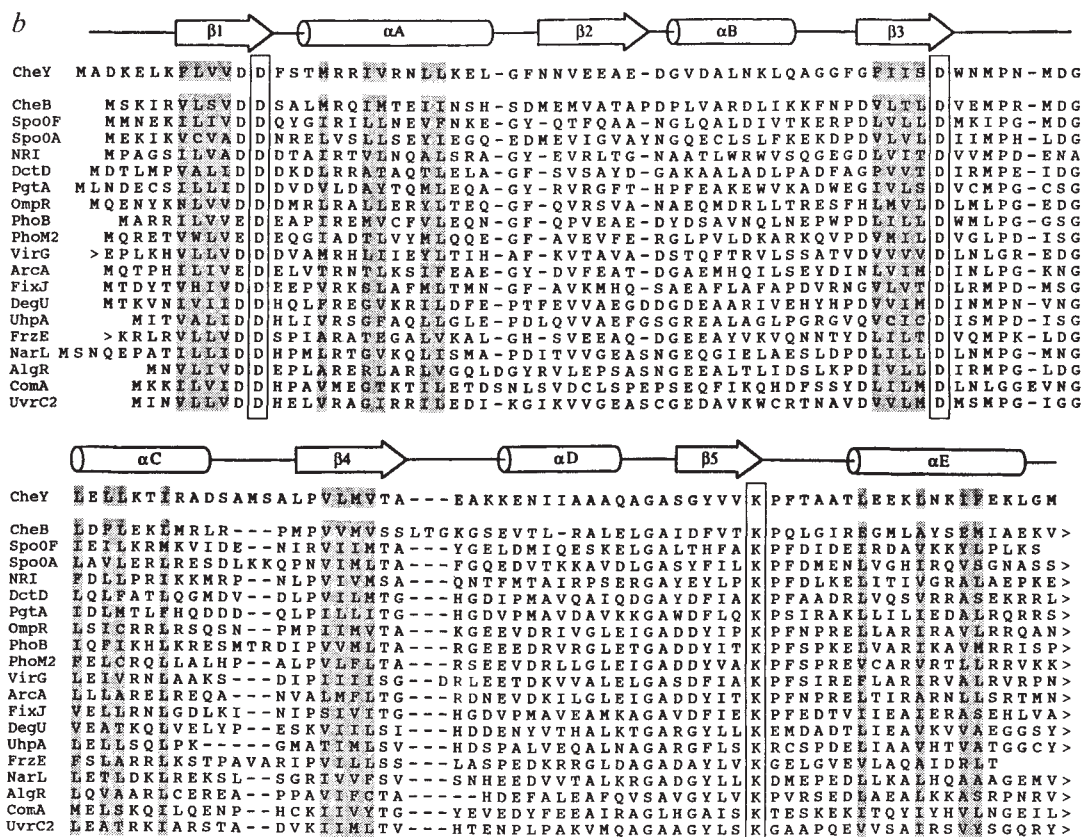


TABLE 1 Standard Gibbs free energy of hydrolysis of phosphorylated amino-acid side chains

Phosphorylated residue	$\Delta G_{\text{hydrolysis}}^{\circ}$ (kcal mol ⁻¹)*		
	Intrinsic†	Proteins‡	Refs
Phosphoserine	-3	-6.5	43
Phosphotyrosine	ND§	-9.5	44, 45
Phosphohistidine	-8 to -10	-12 to -14	46, 47
Phosphoaspartate	-10 to -13	+2	48

* Standard free energies for the reactions: $X-P+H_2O \rightarrow X+P_i$.

† Values estimated from small molecules with similar chemistry⁴⁹: for P-Ser, glucose 6-phosphate and *N*-acetyethanolamine phosphate; for P-His, arginine phosphate and creatine phosphate; for P-Asp, acetyl phosphate and β -aspartyl phosphate.

‡ For P-Asp, protein is Ca^{2+} -ATPase from sarcoplasmic reticulum; for P-His, autophosphorylated CheA, and Enzyme I, HPr, and Enzyme III of the phosphoenolpyruvate:glucose phosphotransferase system; for P-Ser, lysozyme phosphorylated by cAMP-dependent protein kinase; for P-Tyr, immunoglobulin G (IGG) phosphorylated by pp60^{src} and the autophosphorylated EGF receptor.

§ ND, not determined. $\Delta H_{\text{hydrolysis}}^{\circ}$ of free tyrosine *O*-phosphate is -2.8 kcal mol⁻¹, about half that of $\Delta H_{\text{hydrolysis}}^{\circ}$ of ATP⁴⁴.

(P_{II}) also directly regulates the bifunctional enzyme that adds and removes adenylates to control the sensitivity of glutamine synthetase to allosteric ligands^{12,15}.)

NR_{II} is autophosphorylated at a histidine residue, and this phosphoryl group is then transferred to an aspartic acid side chain in the response regulator domain of NR_I (ref. 6). As with EnvZ in the porin regulation system, NR_{II} also shows phosphatase activity^{13,14}. In the presence of P_{II} in its deuridylylated form, NR_{II} increases the rate of phospho- NR_I dephosphorylation^{13,14} thus decreasing transcription of *glnA*. *glnA* is encoded in an operon together with the genes that encode NR_{II} (*glnL*) and NR_I (*glnG*), and so, by activating *glnA* transcription, phospho- NR_I induces its own synthesis as well¹⁰. This positive feedback loop makes the system function as an on-off switch. If nitrogen remains limiting, so that P_{II} is present in its uridylylated form, the high levels of phospho- NR_I also activate transcription of other genes involved in nitrogen assimilation including, in some strains, *nif* genes that are required to fix atmospheric nitrogen¹⁷.

Chemotaxis

Motile bacteria swim toward attractants (generally nutrients such as amino acids, peptides, and carbohydrates) and away from repellents (generally toxic substances)¹⁸. In this case, the histidine kinase component of the regulatory system, CheA, is not a membrane receptor¹⁹. Rather, its activity is controlled by a family of membrane receptors (Fig. 2c) that continuously monitor the chemical composition of the cell's surroundings²⁰⁻²³. These act indirectly, through another protein, CheW, to control CheA kinase activity^{22,24,25}. CheA is autophosphorylated at a histidine residue, and this phosphoryl group is then transferred to an aspartyl residue in the response regulator of the chemotaxis system, the CheY protein²⁶⁻²⁸. Several lines of evidence suggest that phospho-CheY interacts with the flagellar motor to control swimming behaviour¹⁸. CheA, unlike EnvZ and NR_{II} , has no phosphatase activity of its own. Instead, another protein, CheZ, acts as a phosphatase to accelerate the dephosphorylation of phospho-CheY²⁹.

There is a further control mechanism in chemotaxis. The sensitivity of the membrane receptor proteins is controlled by methylesterification at glutamic acid side chains^{30,31}. Receptor demethylation, and consequent desensitization, is catalysed by an enzyme, the CheB methyltransferase, that belongs to the response regulator family²³. The CheB protein is composed of two domains, an N-terminal regulatory domain that accepts phosphoryl groups from CheA^{26,29,32}, and a C-terminal domain

that functions as an esterase to hydrolyse glutamyl methylester groups in the membrane receptor proteins^{33,34}. Phosphotransfer from CheA to CheB stimulates esterase activity, thereby providing a negative feedback loop to attenuate the receptor output^{26,32}. Phospho-CheB is intrinsically unstable, hydrolysing to CheB plus P_i with a half life of only ~ 5 s^{26,29,32}. In a sense the regulatory domain of CheB is a phosphatase that competes with CheY for phosphoryl groups in CheA. The interplay between this phosphatase activity and the receptor methyltransferase activity associated with the C-terminal domain may provide a crucial link between excitation and adaptation^{30,31,35}.

General features

The close mechanistic relationship between phosphorylation in chemotaxis, nitrogen regulation, and regulation of porin expression has been established by the demonstration of cross-talk between these three systems. Using purified components, it has been shown that CheA can phosphorylate NR_I or OmpR to activate transcription from the *glnA* or *ompF* promoters; EnvZ can phosphorylate and activate NR_I and can phosphorylate CheY^{7,36}. Moreover, a hyperactive variant of the nitrogen kinase, NR_{II} 2302, can act in place of CheA to produce tumbling swimming behaviour *in vivo*³⁶.

Members of the response regulator family all share a homologous N-terminal domain of ~ 130 residues (Fig. 1). The three-dimensional X-ray crystallographic structure of one response regulator, the CheY protein, has recently been determined³⁷. CheY is composed of a single α/β -domain with a central five-stranded parallel β -sheet surrounded by five α -helices (Fig. 3a). The aligned sequences of other response regulator domains reveal patterns of similarity which can be interpreted in terms of the CheY structure (Fig. 3b). The proteins all contain clusters of four hydrophobic residues that align with the three central strands ($\beta 1$, $\beta 3$, and $\beta 4$) of the β -sheet in CheY, and all the proteins show a conserved periodicity of hydrophobic residues that in CheY corresponds to the internal faces of α -helices flanking the β -sheet (αA , αC , and αE). The hypothesis that response regulator domains all share a common α/β structure is strongly supported by this conservation of residues that define the hydrophobic core of CheY (Fig. 4a, b).

Three amino acid residues are highly conserved among all members of the response regulator family (Fig. 3b). These residues (Asp13, Asp57 and Lys109) lie on one face of the CheY structure (Fig. 4c and 4d). The two aspartates, together with Asp12 (another conserved residue that is either aspartate or glutamate in all members of the family), are clustered in an acidic pocket at the C-terminal edge of the β -sheet. In α/β proteins, such as certain reductases, kinases, dehydrogenases, and carbohydrate-binding proteins, the ligand-binding site is invariably located in the crevice formed by loops connecting the C-terminal end of β -strands to α -helices lying on opposite sides of the β -sheet³⁸. The conserved aspartates in CheY are located in such a cross-over crevice formed by loops connecting β -strands 1 and 3 to the α -helices A and C that flank opposite sides of the sheet. This conserved acidic pocket is the site of aspartyl phosphorylation^{26,28}.

The three-dimensional structure of a histidine kinase has not yet been determined. A comparison of the amino-acid sequences of these proteins indicates a homologous C-terminal region of ~ 100 residues, as well as sequence similarities surrounding a conserved histidine residue which is thought to be the site of autophosphorylation (Fig. 1). The conserved histidine kinase domain can either function as part of a transmembrane receptor protein (for example, EnvZ) or as a soluble cytoplasmic signal transduction component (such as NR_{II} and CheA). These enzymes are analogous in this respect to the mammalian tyrosine protein kinases that serve similar functions in eukaryotic regulatory systems³⁹.

CheA is phosphorylated at His-48 (ref. 27). Phospho-CheA can be cleaved with proteases to release a phosphorylated

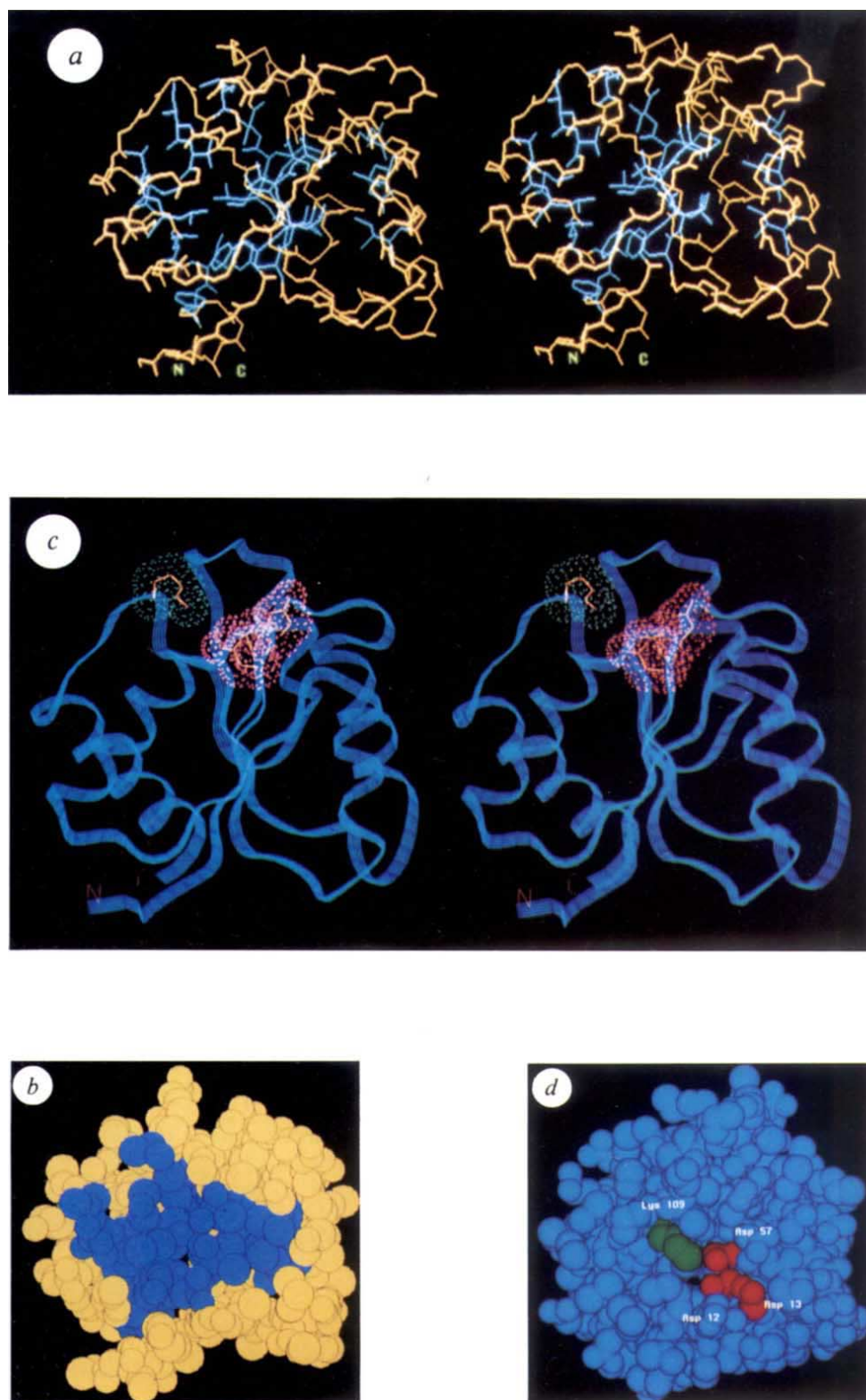


FIG. 4 Residues in CheY that are conserved in response regulator proteins. *a*, Stereo diagram of CheY showing the backbone with conserved hydrophobic core residues in blue (shaded in Fig. 3*b*). The CheY structure is viewed in an orientation similar to that of Fig. 3*a*. *b*, A cross-sectional view of CheY with the hydrophobic core in blue. *c*, Stereo diagram of CheY as in *a*, showing van der Waals' surfaces of Asp 12, Asp 57, and Asp 13 (left to right) in red and Lys 109 in green. These residues comprise the highly conserved active site of the response regulator protein family. *d*, Space filling view of the CheY active site.

N-terminal polypeptide of M_r 18,000 that can still donate its phosphoryl group to either CheY or CheB but is completely deficient in autophosphorylation activity²⁷. The conserved C-terminal sequences are therefore not required for protein-protein interaction between the histidine kinase and response regulator components, nor is this region required to catalyse the transfer of phosphoryl groups from histidine to aspartate side chains. Rather, the conserved kinase domain probably defines the active site for ATP binding and histidine phosphorylation.

Phosphoprotein chemistry and function

The chemistry of phosphorylation at histidine and aspartate residues is quite distinct from the more commonly observed phosphorylations at serine, threonine and tyrosine side chains. The different cellular functions of different types of protein phosphorylation may be understood in terms of the thermodynamics of their formation and hydrolysis (Table 1). The free energy of protein phosphorylation is determined by a combination of the intrinsic free energy of a given phosphoryl-amino acid bond and the associated change in conformational free energy of the protein.

In Table 1 the intrinsic free energies of phosphorylation for the commonly phosphorylated amino acids are compared with the free energies of phosphorylation of the same amino acids when they are residues of proteins. The intrinsic stability of phosphoserine is well suited for the induction of energetically unfavourable conformational changes that act in concert with the effects of non-covalently associated ligands to modulate critical enzymatic activities⁴⁰. Thus, the free energy of hydrolysis of phosphoserines in proteins is higher than that of the free amino acid.

As isolated amino acids, both histidine and aspartate when phosphorylated are 'high-energy' molecules, with the equilibrium well over to the unphosphorylated state. In the proteins, however, there is a striking difference in the energetics of phosphorylation, depending on which amino acid is phosphorylated. For phosphohistidine residues in proteins, the large negative free energy of dephosphorylation is essentially similar to that

for the isolated amino acid, implying that the phosphorylation of this amino acid is almost independent of the protein of which it is a part. This is just what is needed for a protein functioning as a catalytic phosphate donor, with the free energy able to drive phosphorylation of its substrate.

For aspartate phosphorylation, however, the free energy of phosphorylation of the protein is very different from that of the isolated amino acid, implying distinct dependence on the rest of the protein. This is consistent with the phosphate group's interacting with the protein to trigger a transition from one conformational state to another. In the ion-translocating P-type ATPases, aspartyl phosphorylation activates a conformational transition that pumps ions across cell membranes^{41,42}. In the bacterial cytoplasm an analogous phospho-activated switching mechanism regulates motility and controls gene expression.

Discussion

In bacteria, protein phosphotransfer reactions have a central role in the elaboration of adaptive responses to changing environmental conditions. The mechanism of phosphoryl group transfer from histidine to aspartate provides a basis for understanding the sequence similarities that identify two families of bacterial proteins involved in these adaptive responses. Regulation of such responses often involves a pair of proteins, one from each family. Although a common chemical mechanism of communication between these pairs of proteins has been established, many questions remain unanswered. What are the molecular mechanisms that regulate the kinase and phosphatase activities? What is the nature of the response regulator switch mechanism? Do analogous phosphotransfer reactions play a part in eukaryotic signal transduction systems? The answers to these and related questions should provide insight into the molecular logic that underlies information processing within single cells. □

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Limits on the emission of neutrons, γ -rays, electrons and protons from Pons/Fleischmann electrolytic cells

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Emissions of γ -rays from the cold-fusion cells used by Pons and Fleischmann were monitored in Pons' laboratory at the University of Utah by NaI detectors nearly continuously over a five-week period. No evidence of fusion activity was observed above power limits varying between 10^{-12} and 10^{-6} W for the known fusion reactions. In addition, neutron-track detectors indicated an integrated upper limit of approximately 1 emitted neutron per second from any of the cold-fusion cells over a period of 67 hours.

PONS and Fleischmann¹ claim to have achieved 'cold fusion' of deuterium nuclei in electrolytic cells containing palladium cathodes and D_2O (with 0.1 M LiOD) electrolyte, which produced excess heat of the order of a few watts. Their claim was based on the lack of a known electrochemical mechanism for the observed heat excess and the emission of γ -rays and neutrons at levels slightly above background. These latter data have been criticized²⁻⁴, but reports of tritium production in similar cells⁵ and the discrepancy between cell power inputs and outputs in several laboratories⁶ have sustained the controversy despite a large number of negative results⁴.

The known $d+d$ and $d+p$ reactions and their energy yields (Q) are⁷: $d(d, p)t$ ($Q = 4.03$ MeV); $d(d, n)^3He$ ($Q = 3.27$ MeV); $d(d, \gamma)^4He$ ($Q = 23.85$ MeV); $d(d, e^-)^4He$ (internal conversion) ($Q = 23.85$ MeV); $d(p, \gamma)^3He$ ($Q = 5.49$ MeV).

Several weeks after his initial press announcement, Pons allowed us into his laboratory to make independent measurements of any radiation emanating from his operating electrolytic cells. Using equipment that was immediately available, a lead-shielded sodium iodide (NaI) detector (8×4 in.) was installed below his cells (Fig. 1) and collected data nearly continuously for over five weeks in a γ -ray energy range of 0.1–25.5 MeV. In addition, several neutron detectors, which integrated the neutron flux over a period of approximately three days, were placed within the water tank adjacent to the cells; these were made of ^{235}U foils sandwiched between nuclear-track-detecting plastic film.

NaI detection of γ -rays

Four open cells (no gas collection) underwent electrolysis nearly continuously while the NaI detector was collecting data (9 May to 16 June). These cells consisted of D_2O (0.1 M LiOD) electrolyte with platinum anodes; the cathodes were as described in Fig. 1. The cells were run in constant-current mode, and current settings were varied over the five-week interval.

The NaI detector system was placed under the table supporting the water tank and cells, so as not to interfere with other research activities in Pons' laboratory. Any γ -rays produced in

the cells would thus pass through water, water tank and table before being detected by the 8×4 in. NaI detector. The energy-dependent, absolute efficiency for γ -ray detection in the photo-peak (corresponding to complete γ -ray energy conversion within the scintillator) was determined with standard γ -ray sources in a separate laboratory, where an identical configuration of water, tank, table and detector could be installed or removed at will. Radiation limits given below are based on the absolute efficiency for a source at the location of cell 2-1.

Spectral data accumulated by a pulse-height analyser were downloaded to a microcomputer every 0.5 or 1 h (live time) continuously over the five-week observation period, thereby minimizing the effect of integrated background on transient signals. The system's gain was set so that γ -rays in the energy interval 0.1–25.5 MeV were recorded. An aggregate spectrum corresponding to 785 h of operation is shown in Fig. 2a, b.

Individual spectra (1,116), each of 0.5 or 1.0 h live integration time (for a total of 831.0 h of live time), were searched for transient γ -ray signals above background. Background for each

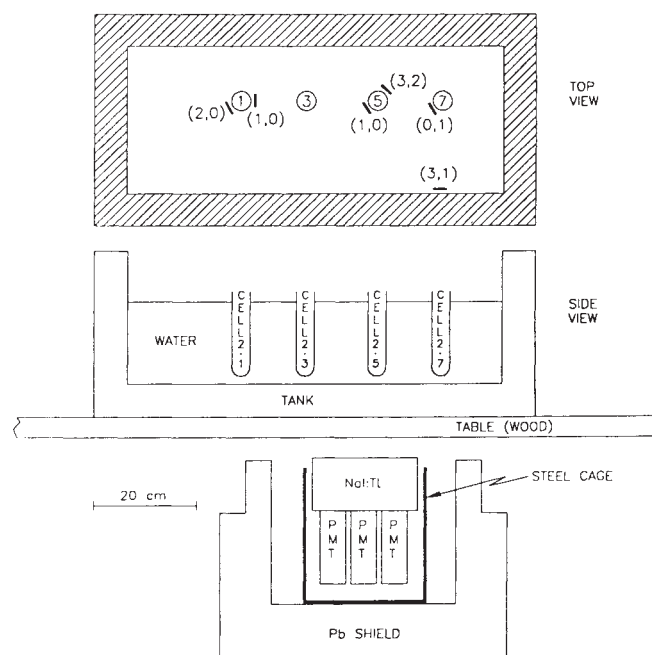
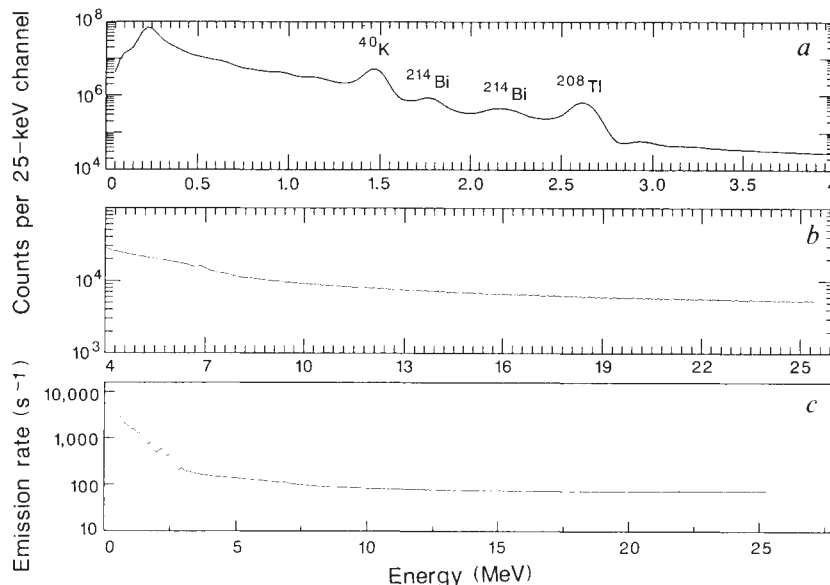


FIG. 1 Side view of the geometry of the electrolytic cells and NaI detector, and top view of the cells with neutron-detecting sandwiches in place (thick-line segments). Cells 2-1, 2-5 and 2-7 have palladium cathodes with diameters/lengths (in cm) respectively of 0.4/1.25, 0.4/10.0 and 0.1/10.0; cell 2-3 has a platinum cathode of dimensions 0.1/10.0. The two numbers (n, m) shown for each sandwich in the top view are the number of fission fragments counted in the plastic film adjacent to the uranium foil (without, with) Cd covers. Only one plastic film per foil was analysed.

FIG. 2 *a, b*, Aggregate γ -ray spectrum for 785 h of live collection time. The dominant background lines are ^{40}K (1.4608 MeV), ^{214}Bi (1.7645 MeV), ^{208}Tl (2.6146 MeV), a backscatter peak at ~ 0.23 MeV, and a broad peak at ~ 2.17 MeV due to two lines, ^{214}Bi (2.1186, 2.2042 MeV). The detector energy resolution is given by $R=0.07E^{-1/2}(1+1.3/E)^{1/2}$, with E in MeV, where the leading factor is the detector's optimal resolution and the additional factor is caused by the presence of noise at the charge-integrating input of the pulse-height analyser's analog-to-digital converter. The integral nonlinearity of the system electronics was found to be $<0.1\%$ over the interval 0.1–3.5 MeV, and 0.2% over the full interval 0.1–25.5 MeV. System gain variations (owing to phototube drift, for example) were monitored and found to be $<3.5\%$ over the full five-week interval and $<0.5\%$ over any 24-h interval. *c*, γ -ray source emission-rate limit against energy for the general γ -ray search over the range 0.1–25.5 MeV (see text). The probability of falsely rejecting a signal at these limits is $<10^{-3}$.



individual spectrum was obtained by averaging several (10–50) successive spectra to form an aggregate spectrum, which was then subtracted from the original spectrum to form a ‘residual spectrum’. This was performed for each individual spectrum, yielding 1,116 residual spectra. Any transient anomalous signals of duration <10 –50 h would then appear in the residual spectra as positive excesses amidst Poisson fluctuations about zero. Signals of longer duration were searched for with even greater sensitivity by performing a similar operation on the collection of aggregate spectra.

To correct for temporal variations in the detector system's gain and pedestal (the pulse-height-analyser channel corresponding to zero energy), the gain and pedestal were determined for each individual spectrum by fitting to the background γ -ray lines. All spectra were then rescaled to a common gain with zero offset before background subtraction, thus minimizing artefacts in the residual spectra arising from gain or zero shifts, or both.

Neutron flux limits: $\text{d(d,n)}^3\text{He}$. Neutrons from the $\text{d(d,n)}^3\text{He}$ fusion channel have an initial kinetic energy of 2.45 MeV and

a mean free path in water of 4.9 cm, which decreases to ~ 0.4 cm as the neutron thermalizes. Because each cell is surrounded by several inches of water, most neutrons emitted would thermalize in the surrounding water bath and generate a 2.22-MeV γ -ray from the $\text{n(p,d)}\gamma$ reaction; Monte Carlo calculations show for a point source of 2.45-MeV neutrons located at cell 2–1 that 62% of the emitted neutrons would be captured by hydrogen in the water bath. The absolute photopeak detection efficiency (for cell 2–1), measured with a Am-Be neutron source, was found to be 3.0×10^{-3} .

The presence of a γ -ray signal above background at 2.2 MeV was sought in each residual spectrum by fitting a gaussian (of central energy 2.2 MeV and variance determined by detector resolution) to the residual spectrum in the energy interval 2.0–2.4 MeV. The frequency distribution of the fitted amplitudes, in units of pW of fusion power, is shown in Fig. 3*a*. Of these 1,114 spectra, the maximum fitted amplitude is 10 pW. In this distribution (and others following) we have excluded two spectra that contained photopeaks due to ^{22}Na and ^{137}Cs sources brought into the laboratory by other personnel.

FIG. 3 Estimated rates for the four d+d fusion reactions. For each of the 1,114 γ -ray spectra obtained from data-collection episodes lasting 0.5–1 h, a residual spectrum was obtained by subtracting an averaged background spectrum from the signal. A fit was then made to each residual by scaling the expected form of the γ -ray spectrum for each reaction; the fit yields a magnitude that can be positive or negative. The figures here are histograms of those 1,114 magnitudes. In *a* and *b*, the rate is expressed in units of fusion power (*a*: pW; *b*: mW); for *c* and *d*, reaction rates are used (*c*: s^{-1} ; *d*: 10^3 s^{-1}). In each case the histogram is approximately a gaussian distribution of amplitudes centred around zero, indicating no measurable rates for any of the fusion reactions.

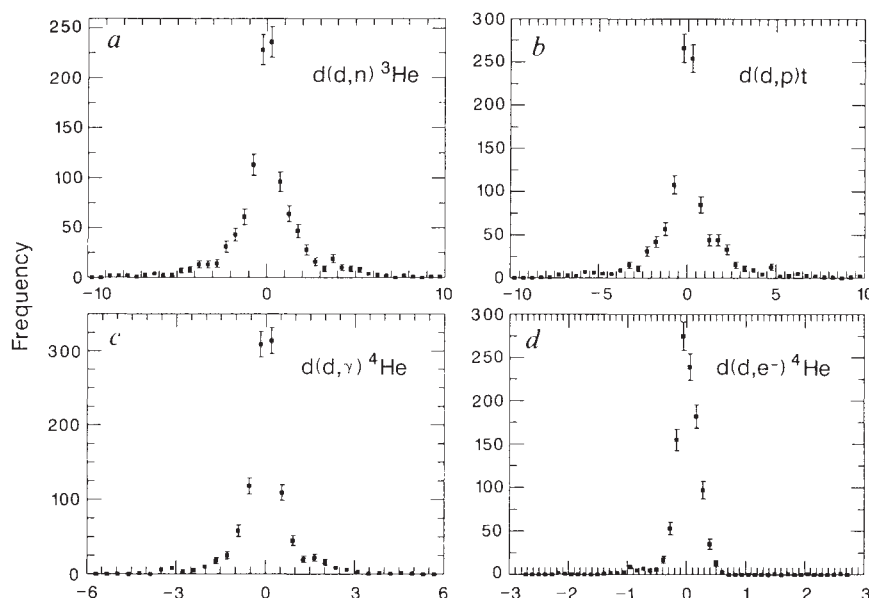


TABLE 1 Radiation yields of 3-MeV protons in palladium electrodes

Isotope (E2 energy) (MeV)	Isotopic fraction	Proton energy (MeV)	Excitation/ μC	NaI detector efficiency η	Pd escape probability
^{104}Pd (0.555)	11.0	3.30	$5.08(0.16) \times 10^5$	2.3×10^{-3}	0.81
		2.40	$4.35(0.17) \times 10^4$		
^{106}Pd (0.513)	27.3	3.30	$7.47(0.18) \times 10^5$	2.3×10^{-3}	0.80
		3.00	$4.00(0.12) \times 10^5$		
		2.70	$1.98(0.06) \times 10^5$		
^{108}Pd (0.433)	26.7	3.30	$1.19(0.03) \times 10^6$	2.2×10^{-3}	0.77
		3.00	$7.02(0.19) \times 10^5$		
		2.70	$3.76(0.08) \times 10^5$		
^{110}Pd (0.374)	11.8	3.30	$1.82(0.04) \times 10^6$	2.1×10^{-3}	0.73
		3.00	$1.11(0.02) \times 10^6$		
		2.70	$6.15(0.13) \times 10^5$		

Proton flux limits: $d(d, p)t$. The fusion channel $d(d, p)t$ ($Q = 4.03$ MeV) has been a leading candidate for the cold-fusion process, both because of reports of excess tritium production⁵ and because its reaction products come to rest within the palladium electrode (the range of a 3-MeV proton in palladium is ~ 30 μm ; ref. 8), thereby presumably avoiding the paradox of watts of fusion power being generated without observed particle emissions. In fact, a strong and distinct γ -ray signature exists for this reaction: the 3.02-MeV protons cause Coulomb excitation of the even-even isotopes of Pd, whose radiative de-excitations, between 0.37 and 0.56 MeV, are detectable by the NaI detector with efficiencies η given in Table 1. This table, adapted from ref. 9, lists for each Pd isotope its E2 (electric quadrupole) γ -ray energy and thick-target radiation yield (excitations per microcoulomb of protons) for 100% isotopically enriched samples, along with photopeak detection efficiency assuming cell 2-1 as the source, plus a factor accounting for absorption of the E2 γ -ray within the Pd cathode. Figure 4 shows a NaI γ -ray spectrum from a 3-mm target of natural Pd exposed to a beam of 3.02-MeV protons from a Van de Graaf accelerator (W. Schier, personal communication). Peaks at 0.37, 0.43 and 0.51 MeV are observed with their expected strengths.

Convolving these line strengths with the resolution of the NaI detector, and using the efficiency factors from Table 1, a theoretically generated spectrum was scaled to optimally fit the 0.362–0.613-MeV region of each residual spectrum. The frequency distribution of the 1,114 fitted scaling factors (in units of mW of fusion power) is shown in Fig. 3b. None of the scaling factors exceeds 10 mW. This limit is comparable to the sensitivity of the calorimetric measurements made on these cells.

More stringent limits can be placed on this channel by recognizing that the tritium, emitted with kinetic energy of 1.0 MeV, can initiate the $t(d, n)^4\text{He}$ reaction. By integrating the energy-dependent reaction cross-section¹⁰ over the range of the tritium within the Pd, we obtain a $d+t$ fusion probability of 4×10^{-5} per emitted tritium, assuming a 1:1 d: Pd ratio in the lattice. (We note that the probability p that a 1.0-MeV triton will produce a neutron within a deuterated palladium lattice via the reaction $t(d, n)^4\text{He}$ can be expressed as a function of r , the deuterium-to-palladium ratio (by number): $p = a_0 + a_1 r + a_2 r^2$, where $a_0 = 9.63 \times 10^{-8}$, $a_1 = 4.48 \times 10^{-5}$ and $a_2 = -5.40 \times 10^{-6}$. This expression, based on nuclear cross-sections from ref. 10 and hydrogen stopping power from ref. 17, is accurate to $\leq 10\%$ and is valid for $0.4 \leq r \leq 1.5$.) Monte Carlo calculations show that 39% of the resulting ~ 14 -MeV (centre-of-mass) neutrons will thermalize and be captured by protons within the water tank, yielding a 2.2-MeV γ -ray signal. From the absence of this signal, we obtain an upper limit of the fusion power amplitude of 0.4 μW .

γ -ray flux limits: $d(d, \gamma)^4\text{He}$, $d(p, \gamma)^3\text{He}$, monoenergetic γ . A search was performed for 23.85-MeV γ -rays from $d(d, \gamma)^4\text{He}$ by fitting the expected line profile of a 23.85-MeV γ -ray to the residual spectra over the energy interval 23.6–24.1 MeV. Figure 3c shows the resulting distribution of fitted source emission rates; the

maximum fitted rate is 5 s^{-1} , corresponding to a fusion power of 20 pW. A similar search was performed for 5.49-MeV γ -rays from $d(p, \gamma)^3\text{He}$; the maximum fitted source emission rate of 10 s^{-1} corresponds to a fusion power of ~ 10 pW.

A general search was also performed throughout the entire 0.1–25.5-MeV energy interval for possible γ -ray lines above background in each residual spectrum. Candidates were required to have at least one channel with counts in excess of 3σ (σ being the propagated Poisson error for that channel's count) and a summed excess of 9σ in the adjacent channels spanning the full width at half maximum of a γ -ray peak at the sampled energy. The only candidates found were due to imperfect rescaling of the spectrum's gain and zero relative to averaged background; these were identified by the presence of adjacent positive and negative excursions of equal magnitude, with a zero-crossing at a known background peak position. No other candidate γ -ray lines were found. Allowing for detector efficiency, this yields the relationship between γ -ray emission-rate limit and energy (for cell 2-1) shown in Fig. 2c.

Internal-conversion electron flux limits: $d(d, e^-)^4\text{He}$. Even though nuclear de-excitation by internal conversion is greatly suppressed relative to radiative de-excitation for low-atomic-mass nuclei and for photon energies much greater than the electron mass, it has been suggested that cold fusion may in fact be

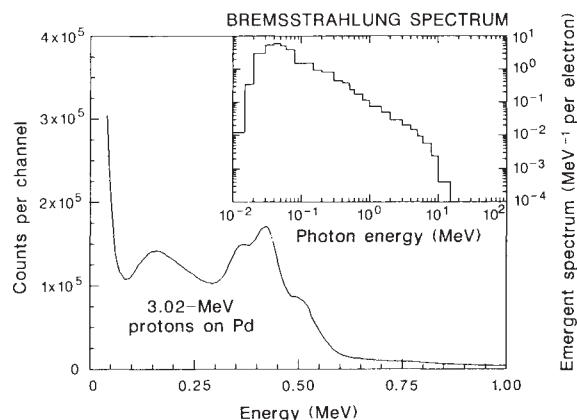
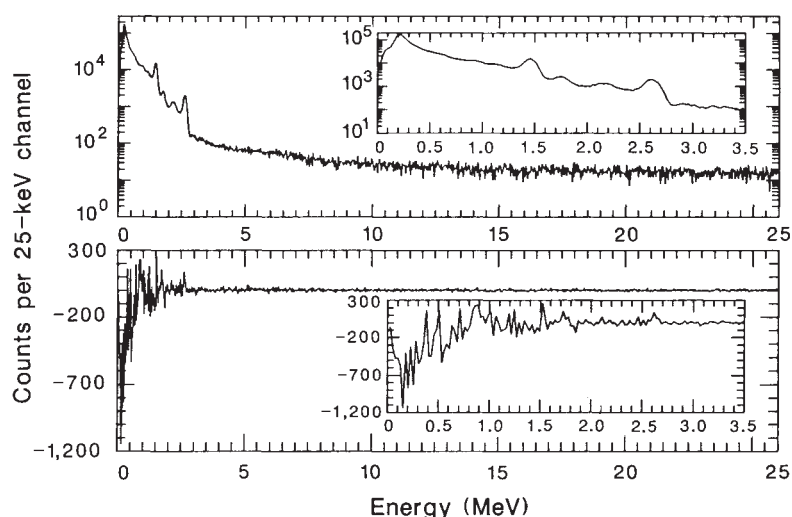


FIG. 4 Electric-quadrupole (E2) γ -ray lines from the isotopes of Pd, excited by 3.02-MeV protons, as measured by a 3×3 in. NaI detector. Three lines at 0.37, 0.43 and 0.51 MeV constitute a clear γ -ray signature for the reaction $d(d, p)t$ (a fourth line at 0.56 MeV from ^{104}Pd is too weak to be seen here). Their collective backscatter peak is at ~ 0.16 MeV. Inset: The emergent bremsstrahlung spectrum for an electron emitted at 20 MeV at the centre of a cylinder of H_2O , of height 36 cm and diameter 36 cm. The normalization is for a single electron, with the γ -ray flux integrated over the cylinder surface; the area under the spectrum corresponds to 1.1 photons per electron, including a small δ -function contribution of 0.511-MeV annihilation quanta not shown in the figure. This spectrum was obtained using the Monte Carlo code ETRAN¹⁵, which treats the coupled electron-photon transport using a recently developed set of bremsstrahlung cross-sections¹⁶.

FIG. 5 *a*, γ -ray spectrum accumulated over a 2.5-h interval that included a ~ 2 -h period during which cell 2-1 was observed to boil the D_2O electrolyte. The inset is an expansion of the low-energy end of the spectrum. *b*, Residual spectrum after subtraction of the spectrum accumulated for the preceding 2.5 h. The negative excess at low energy is due to a slight (0.1%) gain shift that occurred during this 5-h period (these spectra were not rescaled).



occurring via internal conversion¹¹, which would produce an electron that carries off 23.8 MeV in kinetic energy.

Figure 4 shows the calculated bremsstrahlung spectrum emerging from a point-isotropic source of 20-MeV electrons located at the centre of a cylinder of water 36 cm in diameter (~ 4 MeV are assumed to have been lost within the Pd cathode¹²). The spectrum, normalized to one source electron, has an integrated area of 1.1 photons per electron. This bremsstrahlung spectrum, modified by detector efficiency, was scaled to obtain an optimal fit to each residual spectrum, giving a best-fit number of bremsstrahlung photons for each spectrum. Figure 3*d* shows the frequency distribution of the electron emission rate corresponding to the fitted bremsstrahlung photon number. The maximum fitted electron emission rate, 2.6×10^3 electrons per second, corresponds to a fusion power level of 10 nW.

Neutron detection with nuclear track detectors

A significantly lower limit than the 10 pW discussed above on the mean neutron production rate during a 67-h interval (16–19 May) was obtained with neutron-detecting sandwiches made of ^{235}U -enriched (80%) uranium foils and nuclear-track-detecting plastic, Lexan polycarbonate. Six sandwiches were installed in the water tank containing the cells (Fig. 1); each consisted of two uranium foils, each with an area of 1.4 cm^2 and mass $\sim 0.078\text{ g}$, held between two pieces of 0.01-in.-thick Lexan polycarbonate. One of the two foils was shielded against slow neutrons ($<0.5\text{ eV}$) by two cadmium covers.

The fission capture cross-section for ^{235}U is 1.4 barn at 2.5 MeV, increasing to 580 barn at thermal energies. Some of the neutrons emitted at 2.45 MeV from the cells will be captured by ^{235}U nuclei during their diffusion within the water bath. The fission fragments have a typical range of a few micrometres in U; those that escape the foil enter the plastic film and rupture polymer bonds, thereby creating a nuclear track that can be viewed microscopically after chemical etching with NaOH (ref. 13). (The track registration threshold of Lexan is such that the numerous α particles produced by the ^{238}U component of the U foils do not produce visible tracks.)

The absolute neutron detection efficiency was measured with a ^{252}Cf source in a large water tank, with foil sandwiches placed relative to the source identically to those in Pons' laboratory, and was found to be 1.3×10^{-5} fission tracks per emitted neutron for a foil with no Cd cover; for those foils with Cd covers, the efficiency was a factor of about three lower.

Fission track counts for each sandwich's pair of foils are shown in Fig. 1, the first number being the count for the foil without a Cd cover and the second being that with. There was no indication of an excess neutron signal from any of the cells;

in fact, the control sandwich, adjacent to the water-tank wall, registered one of the highest fission-track counts, these being a measure of the dominant background source, cosmic-ray neutrons¹⁴. If we assume zero background, however, the fission track counts at cell 2-5 correspond to a mean neutron emission rate of 0.8 s^{-1} over the 67-h integration period, with a 99% confidence-level upper limit on neutron emission rate from cell 2-5 (based on counts in foils without Cd covers) of 1.8 s^{-1} , corresponding to a fusion power level of 0.9 pW.

Pons has informed us that "no neutron detectors were ever placed in a tank when a cell in that tank was generating excess enthalpy".

Fusion limits and excess heat production

During the 831 h (live time) of monitoring γ -ray emissions from electrolytic cells in Pons' laboratory, no evidence was seen of radiation from any known d+d (or p+d) fusion reaction. The upper limits placed on power from these reactions range between 10^{-12} and 10^{-6} W , which are many orders of magnitude lower than the sensitivity of the calorimetric measurements made by Pons' group; therefore, if a heat excess were to have occurred during our period of observation, one could conclude that no known fusion process contributed significantly to that excess.

At one point, the D_2O in cell 2-1 was observed to boil for ~ 2 h. Figure 5*a* shows an aggregate γ -ray spectrum for the 2.5 h that included this boiling episode, and Fig. 5*b* is the residual spectrum after subtraction of the previous 2.5 h of data. No spectral features of fusion are present in this residual spectrum. After completing our analysis, we were informed that we should not "reference these events as being due to release of excess thermal energy" (S. Pons, personal communication), because this boiling event may very well have a conventional explanation. Unfortunately we have not received any numerical data on excess heat production during the 831 h of our monitoring, so we are not able to correlate the absence of nuclear signatures with the presence of anomalous heat.

We were told, however, that "there was a two-hour segment in which there was excessive thermal release from cell 2-1... Unfortunately, your computer and detector were not under power at that time since they had not been reset from a power failure which had occurred in the lab" (S. Pons, personal communication). Although 48 h of data were indeed lost because of a lightning strike, we can nevertheless estimate mean upper limits for fusion power of $\sim 10^{-2}\text{ W}$ for d(d, p)t and 10^{-6} W for d(d, n) ^3He during this 2-h episode, because a fraction of the neutrons produced from these reactions would activate the ^{23}Na in the NaI detector, producing ^{24}Na . As ^{24}Na decays with a

15.0-h half-life, a spectral signature of a neutron burst would be present even several days after the burst. None was observed, leading to the conclusion that neither the $d(d, p)t$ nor the $d(d, n)^3\text{He}$ reaction was responsible for this anomalous heat burst.

In addition, we later learned that a low-level, d.c. heat excess was observed during our monitoring period (S. Pons, EPRI Conference, University of Utah, 16 August 1989); if this is the case, this excess did not originate from known nuclear processes. □

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DNA cleavage catalysed by the ribozyme from *Tetrahymena*

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An RNA enzyme derived from the self-splicing intervening sequence of *Tetrahymena thermophila* catalyses sequence-specific cleavage of an oligodeoxyribonucleotide substrate. Compared with RNA, the DNA substrate is bound very weakly and is cleaved very slowly, revealing the importance of the RNA 2'-hydroxyl group in both the binding and chemical steps. The finding that catalysis by RNA can extend to DNA substrates indicates new possibilities for the transposition of intervening sequences and for the design of DNA cleavage agents with novel sequence specificities.

ALTHOUGH catalytic RNAs, or ribozymes, have received much attention^{1–4}, they have been generally maligned as having poor catalytic efficiency and limited substrate repertoire. But the *Tetrahymena* ribozyme increases the rate of hydrolysis of RNA 10¹⁰ times over the estimated uncatalysed rate, well within the range of rate increases achieved by protein enzymes⁵. Also, this ribozyme cleaves essentially every substrate molecule it binds (D.H. and T.R.C., manuscript submitted) and has therefore attained catalytic perfection as defined by Albery and Knowles⁶.

With respect to the versatility of substrate choice, it seemed that ribozymes were restricted to RNA substrates. But DNA substitutes for RNA in a stoichiometric addition reaction that results in reopening of the circular form of the *Tetrahymena* intervening sequence (IVS) RNA⁷. We now report that the *Tetrahymena* ribozyme catalyses the sequence-specific cleavage of single-stranded DNA, with multiple turnover.

Characterization of the DNA cleavage reaction provides new insight about the nature of substrate-ribozyme interactions.

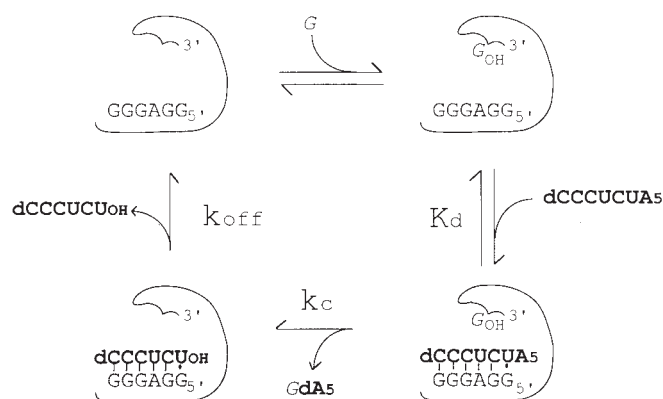
Equilibrium binding of the DNA substrate is about 10⁴-fold weaker than binding of the analogous RNA, and the rate of chemistry with bound substrate is decreased by about another 10⁴-fold. Therefore, one or more of the 2'-hydroxyl groups of the RNA substrate is involved in binding and catalysis. Despite these large differences, the maximum rate during steady-state turnover is only about 10-fold lower for the DNA substrate, because there is a change in the rate-limiting step when DNA is substituted for RNA.

The ribozyme cleaves DNA

The ribozyme used in this study is the L-21 ScaI RNA, a shortened form of the *Tetrahymena* IVS⁸. This ribozyme is an endonuclease that cleaves RNA substrates directly after sequences resembling the 5' exon, by means of attack by guanosine; the analogous reaction for a DNA substrate is shown in Fig. 1. The endonuclease reaction is an intermolecular version of the first step of self-splicing (Fig. 2).

The DNA substrate $d(p^*\text{CCCUCUA}_5)$ (p^* denotes [³²P]/phosphate) was synthesized with dU residues to provide a strict DNA version of the RNA substrate studied previously. This DNA substrate is cleaved specifically at the same position as the analogous RNA substrate (Fig. 1). The product (Fig. 3a, lane 4) co-migrates with $d(p^*\text{CCCUCU})$ (lane 3) and with the appropriate product from P1-nuclease digestion of the DNA substrate (lane 1). Mg^{2+} is required (lane 7), ATP cannot substitute for G (lane 6), and GTP can substitute for G (see below), each of which is also a property of the reaction with RNA substrates. A small amount of product is formed in the absence of added G, but only in the presence of ribozyme and Mg^{2+} (product seen in lanes 5 and 6, but not in lanes 7 and 8 in longer exposures; data not shown). This is analogous to the slow hydrolysis of RNA substrates that occurs in the absence of G (D.H. and T.R.C., manuscript submitted). Similar experiments with $d(p^*\text{CCCUCUA})$ also gave specific cleavage between U and A dependent on the presence of the ribozyme (data not shown).

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Covalent addition of the guanosine cofactor to the 3' product fragment as outlined in Fig. 1 was investigated using a DNA substrate that was 3' end-labelled (dCCCUCUA₅p*3'dA; Fig. 3b). The product from the reaction with GTP (lane 11) migrates faster than that from the reaction with G (lanes 3 and 5), as expected from covalent attachment of GTP and G and the greater negative charge of the GTP product: pppGA₅p*3'dA versus GA₅p*3'dA. After treatment with ribonuclease T1, which cleaves after guanosine residues⁹, the products from the reactions with G and GTP (lanes 4, 6 and 12) both co-migrated with A₅p*3'dA (lanes 2 and 9). This shows that G and GTP attack the substrate at the U–A bond, as depicted in Fig. 1.

The DNA endonuclease reaction is catalytic. For example, a reaction mixture with 25 μ M DNA substrate (dCCCUCUA₅) and a trace of 5'-end-labelled substrate was incubated with 0.55 μ M ribozyme and 800 μ M G for 20 h (see Fig. 4 for conditions). Five per cent of the labelled DNA was converted to the product, corresponding to slightly more than two turnovers.

Steady-state kinetic parameters

The second-order rate constant for reaction of free ribozyme and substrate, $k_{\text{cat}}/K_m = 400 \text{ M}^{-1} \text{ min}^{-1}$, (k_{cat} is the catalytic constant, and K_m is the Michaelis constant) was determined from experiments with ribozyme in excess of labelled DNA substrate (Fig. 4). The value for the inhibition constant of $K_i = 30 \mu\text{M}$ for dCCCUCUA₅ was determined from inhibition by 1–320 μM DNA substrate of the reaction of ³²P-labelled RNA substrate (r(p*G₂CCCUCUA₅)) with the ribozyme in excess, but at a concentration $\ll K_m$ (20 nM ribozyme and ~ 1 nM

FIG. 1 The endonuclease reaction of the L-21 Scd1 ribozyme with a DNA substrate (bold). Details of the reaction mechanism are analogous to those of the corresponding RNA reaction³². Because the equilibrium for the overall reaction in solution is expected to be near unity for this phosphotransfer reaction, all steps are presumably reversible. The binding of G before the oligonucleotide substrate is shown for simplicity; but experiments with RNA substrates show that the two binding sites are essentially independent, so that either order of substrate addition can occur (D.H. and T.R.C., manuscript submitted). The 5' sequence of the ribozyme (shaded), called the 5' exon-binding site, is responsible for recognition of the substrate by base pairing^{8,32–37}.

RNA substrate; K_m for r(p*G₂CCCUCUA₅) = 300 nM; D.H. and T.R.C., manuscript submitted). These conditions give an observed K_i that is equivalent to K_m for the added deoxy inhibitor. This is explained in brief as follows. The reaction of the labelled RNA substrate is first-order with respect to free ribozyme. Therefore, the extent of slowing of the reaction on the addition of inhibitor reflects directly the amount of ribozyme that has bound DNA. This amount is dictated by the K_m of the DNA substrate¹⁰. The rate of reaction of the labelled RNA substrate decreases to zero with increasing concentrations of the DNA substrate, showing that the inhibition is competitive as expected for binding of the RNA and DNA substrates at the same site on the ribozyme.

The values of $k_{\text{cat}}/K_m = 400 \text{ M}^{-1} \text{ min}^{-1}$, and $K_m = 30 \mu\text{M}$, give a k_{cat} of 0.01 min^{-1} . The k_{cat}/K_m is about 10^5 -fold lower and k_{cat} about 10-fold lower than the values for the corresponding RNA substrate, r(G₂CCCUCUA₅) (Table 1). Note that the RNA substrate has two G residues at its 5' terminus to facilitate its transcription by T7 RNA polymerase¹¹. Removal of these G residues from the product oligonucleotide by ribonuclease T1 has only a minor effect on the binding constant, lowering it by about fourfold (D.H. and T.R.C., manuscript submitted). Similarly, the DNA product with two dG residues added to its 5' terminus has the same binding constant, within about twofold, as the product oligonucleotide shown above (data not shown).

DNA binds weakly and is cleaved slowly

The following analysis shows that the large change in k_{cat}/K_m and relatively small change in k_{cat} with the DNA substrate

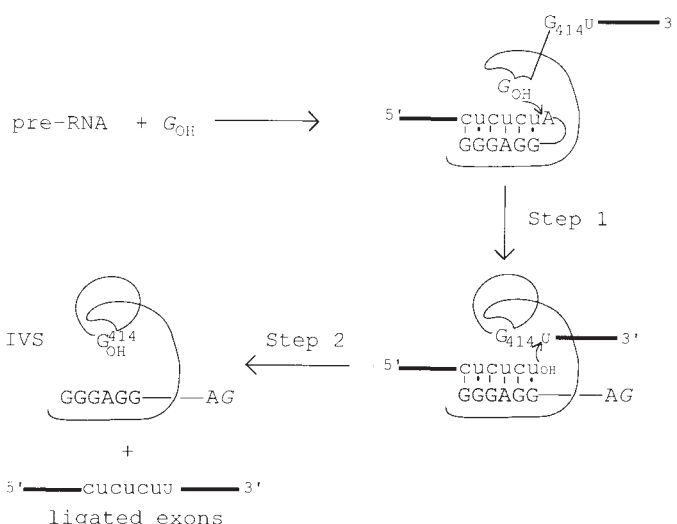


FIG. 2 Pathway for self-splicing of *T. thermophila* pre-ribosomal RNA^{5,38,39}. The 413-nucleotide IVS is excised in two transesterification steps. In the first step, guanosine (or GTP) attacks the 5' splice site so that the bond at this junction is cleaved and G is covalently attached to the 5'-end of the IVS. The nucleotides preceding the 5' splice site are recognized by base pairing to the 5' exon-binding site, a portion of the internal guide sequence^{34–37}. This same binding motif is used in the endonuclease reaction described in Fig. 1. In the second step of splicing, the 3'-hydroxyl group at the end of the 5' exon attacks the 3' splice site after G₄₁₄ to give exon ligation and excision of the IVS.

TABLE 1 Comparison of the kinetic parameters for cleavage of DNA and RNA substrates

	k_{cat}/K_m ($\text{M}^{-1} \text{min}^{-1}$)	k_{cat} (min^{-1})	K_m (μM)	K_d (μM)	k_c^* (min^{-1})
DNA (CCCUCUA ₅)	400	0.01	30	30	0.01
RNA (G ₂ CCCUCUA ₅)	10^8	0.1	0.001	0.002	~ 350
RNA/DNA ratio	3×10^5	10	$1/(3 \times 10^4)$	$1/(2 \times 10^4)$	4×10^4

Reactions were carried out at 50 °C in 50 mM MES buffer, pH 7.0 (at 25 °C), and 10 mM MgCl₂. The data for the DNA reaction are described in Fig. 4 and the text, and the data for the RNA reaction are described by us elsewhere (D.H. and T.R.C., manuscript submitted). As described in the text, the 5'-terminal G residues have little effect on binding for the RNA or DNA.

* The rate constant k_c is that for the chemical conversion of the E~G~S ternary complex (Fig. 1).

replacing the RNA substrate result from large decreases in the binding affinity and the rate of the chemical conversion for the DNA substrate. These large decreases are not fully expressed in k_{cat}/K_m and k_{cat} because there is a change from rate-limiting binding (k_{cat}/K_m) and product release (k_{cat}) with the RNA substrate, to rate-limiting chemistry with the DNA substrate.

Inhibition of the ribozyme by the DNA product, d(CCCUCU), performed as outlined above for determination of K_i for d(CCCUCUA₅), gave a K_i of 20 μM . (Reactions were performed with 1–230 μM d(CCCUCU).) At the high concentrations of d(CCCUCU) the observed rate of reaction approached zero, showing that the inhibition is competitive. The value of K_i is equivalent to K_d because the inhibition represents removal of free ribozyme, which follows the equilibrium: $\text{E} + \text{P} \rightleftharpoons \text{E} \sim \text{P}$ ($K_d = [\text{E}][\text{P}]/[\text{E} \sim \text{P}]$), where E, P and E~P denote the ribozyme, the oligonucleotide product, and the complex that they form, respectively, and the brackets denote concentrations.

The binding of the DNA product is about 10^4 -fold weaker than that of the analogous RNA product (K_d for r(pppG₂CCCUCU) = 1 nM; D.H. and T.R.C., manuscript submitted). Therefore the DNA product should dissociate faster than the RNA product. Dissociation of the RNA product,

r(G₂CCCUCU), is rate-limiting for k_{cat} so that k_{cat} for the DNA reaction would be greatly increased if product dissociation were rate-limiting. But k_{cat} is decreased by about 10-fold with the DNA substrate. This presumably indicates that there is a change to a rate-limiting step involving the conversion of the ternary complex to products. This is presumably the chemical step (with rate constant k_c ; Fig. 1). The value of $k_c (= k_{\text{cat}}) = 0.01 \text{ min}^{-1}$ for the DNA substrate is about 10^4 -fold smaller than k_c for the RNA substrate (Table 1).

With subsaturating oligonucleotide substrate and saturating G, there is a second-order reaction of the E-G complex and S. For the RNA substrate, binding is rate-limiting; the value of $k_{\text{cat}}/K_m = 10^8 \text{ M}^{-1} \text{ min}^{-1}$ for this reaction (Table 1) is essentially equal to the rate constant for formation of helices between oligonucleotides^{12,13}. By contrast, the value of $k_{\text{cat}}/K_m = 400 \text{ M}^{-1} \text{ min}^{-1}$ for the DNA substrate is much smaller than rate constants for helix formation. The strong implication is that binding of the DNA substrate is not the rate-limiting step, so that the subsequent chemical conversion is rate-limiting. This can also be shown by another quantitative argument. The value of $k_c = 0.01 \text{ min}^{-1}$ for the chemical conversion with the DNA substrate is much smaller than k_{off} (the rate constant for the

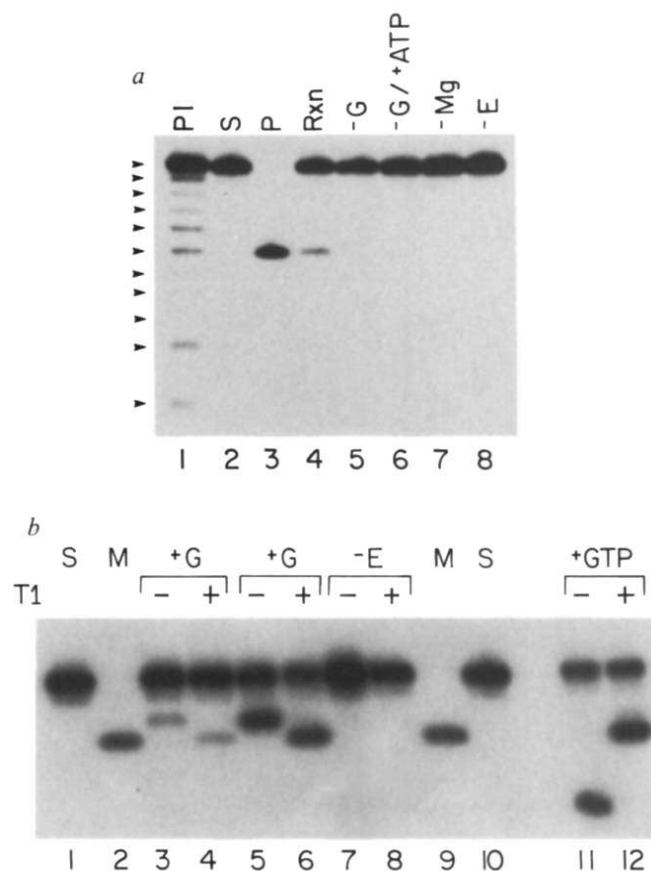


FIG. 3 Endonuclease reaction of a DNA oligonucleotide substrate catalysed by the ribozyme. *a*, Identification of the 5' product fragment: d(p*CCCUCUA₅) ($\sim 2 \text{ nM}$, 5' end labelled with ^{32}P), was incubated with 1 μM L-21 Scal ribozyme for 15 h at 50 °C in the presence of 800 μM G, 50 mM MES, pH 7.0, and 10 mM MgCl₂ (lane 4, Rxn denotes reaction; without G (lane 5); with 800 μM ATP replacing the G (lane 6); without MgCl₂ (lane 7); and without ribozyme (lane 8). Lane 2 shows the substrate, d(p*CCCUCUA₅); lane 3 shows the putative product, d(p*CCCUCU); lane 1 shows the products from digestion of the substrate by 0.2 U μL^{-1} P1 nuclease in 1 mM Tris buffer, pH 7.5, 2 mM ZnCl₂, 0.1 mM EDTA, and 0.1 $\mu\text{g} \mu\text{L}^{-1}$ transfer RNA for 60 min at 37 °C. Ribozyme reactions were initiated by addition of the DNA substrate after 10-min preincubation of the ribozyme in buffer and MgCl₂ at 50 °C. Reactions were quenched by EDTA and urea at 0 °C and electrophoresed on a 20% denaturing polyacrylamide gel⁸. The ribozyme was prepared as described previously⁸. DNA oligonucleotides were synthesized on an Applied Biosystems DNA Synthesizer; dU on a derivatized CPG support (American Bionetics) was used for oligonucleotides with a 3'-terminal dU residue. *b*, Identification of the 3' product fragment: d(CCCUCUA₅p*3'dA) ($\sim 2 \text{ nM}$) was incubated for 4 h with 4 μM L-21 Scal ribozyme, 50 mM MES, pH 7.0, and 10 or 100 mM MgCl₂; one aliquot was quenched with EDTA and urea and another aliquot was treated with 0.05 U μL^{-1} ribonuclease T1 for 1 h at 37 °C. Denaturing PAGE was performed as in *a*. Lanes 3 and 4, reaction with 1 mM G and 10 mM MgCl₂; lanes 5 and 6 reaction with 1 mM G and 100 mM MgCl₂; lanes 11 and 12 reaction with 1 mM GTP and 100 mM MgCl₂. Lanes 7 and 8, from incubation with 1 mM G and 100 mM MgCl₂ in the absence of ribozyme. Lanes 1 and 10, substrate, d(CCCUCUA₅p*3'dA); lanes 2 and 9, expected product after ribonuclease-T1 treatment, d(A₅p*3'dA) (M). Both d(CCCUCUA₅) and dA₅ were labelled at their 3' end with p*3'dA by reaction with [α - ^{32}P]3'dATP catalysed by terminal transferase. No product was observed when G or MgCl₂ was omitted from reaction mixtures containing the ribozyme (data not shown).

dissociation of substrate) = 0.2 min^{-1} for the RNA substrate (D.H. and T.R.C., manuscript submitted), and k_{off} for the DNA substrate should be even larger because of the lower affinity of the DNA oligonucleotides. The large value of k_{off} relative to k_c for the DNA substrate means that this substrate binds and dissociates many times before it reacts. Therefore, K_m is expected to equal K_d for the DNA substrate, and the rate-limiting step is presumably the chemical conversion, as with saturating substrate. The similar values of $K_d = 30$ and $20 \text{ } \mu\text{M}$ for the DNA substrate and product, respectively, and for the corresponding RNA oligonucleotides ($K_d = 2$ and 1 nM for $r(\text{G}_2\text{CCCUCUA}_5)$ and $r(\text{G}_2\text{CCCUCU})$, respectively; D.H. and T.R.C., manuscript submitted) are consistent with the assignment of $K_m = K_d$ for the DNA substrate.

The 2'-hydroxyl in binding

Comparison of the DNA and RNA reactions reveals the importance of the 2'-hydroxyl group in binding and in catalysis. RNA oligonucleotides such as $r(\text{G}_2\text{CCCUCU})$ bind to the ribozyme about 10^4 -fold (6 kcal mol^{-1}) stronger than expected for simple helix formation (D.H. and T.R.C., manuscript submitted). By contrast, the DNA oligonucleotide binds much less strongly (about 10^4 -fold) than the RNA oligonucleotide with the same sequence. This difference contrasts with the small difference in the stability of several RNA-RNA and RNA-DNA helices¹⁴⁻¹⁶. Therefore the DNA oligonucleotide binds to the ribozyme with roughly the affinity expected for a simple helix, indicating that one or more 2'-hydroxyl groups could be involved in tertiary interactions that provide additional stabilization with the RNA oligonucleotide.

The role of the 2'-hydroxyl group in binding could be direct

(for example, hydrogen-bonding or Mg^{2+} coordination) or indirect (for example, favouring the correct helix geometry). The simplest model invokes a direct interaction of the ribozyme with a 2' hydroxyl of the RNA oligonucleotide. It is tempting to propose that tertiary interactions involve the 2' hydroxyl of the conserved U preceding the reaction site; substitution of other bases at this position greatly slows *G* addition in the first step of self-splicing and in a related reaction^{17,18}. Alternatively, there could be an unfavourable helix geometry with the DNA substrate. Although RNA-RNA and RNA-DNA helices both tend to be of the A-form, there are some differences in conformation¹⁹⁻²². In addition, it remains possible that a different helical form occurs on the ribozyme that is much more easily adopted by an RNA-RNA duplex.

Similar conclusions about the importance of the 2' hydroxyl were obtained from the study of a related reaction, the linearization of a circular form of the *Tetrahymena* IVS by $d(\text{CT})$, $r(\text{CU})$, and the mixed ribo-deoxy dinucleotides⁷. Based on comparisons of these substrates and the assumption that $K_m = K_d$, it was suggested that the 2'-hydroxyl moieties are involved in binding the IVS.

The 2' hydroxyl in catalysis

The chemical step of the DNA reaction is about 10^4 -fold slower than the chemical step of the analogous RNA reaction (Table 1). This could indicate that the 2' hydroxyl has an increased role in stabilizing the transition state beyond its role in ground-state binding. But the possibility cannot be excluded that there is destabilization of the DNA-ribozyme duplex (Fig. 1) in the transition state from interactions with the active site that are not present in the ground state; in the ground state this helix

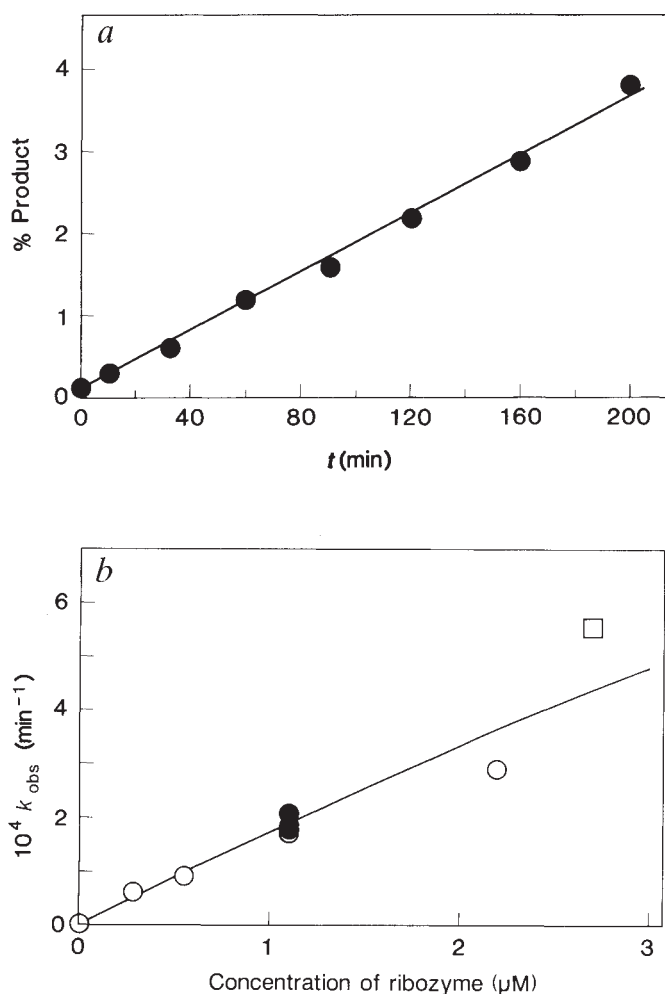


FIG. 4 Determination of k_{cat}/K_m for the DNA endonuclease reaction with $d(p^*\text{CCCUCUA}_5)$. *a*, Time course for formation of product ($d(p^*\text{CCCUCUA}_5)$) with $1.1 \text{ } \mu\text{M}$ ribozyme, $800 \text{ } \mu\text{M}$ G, 50 mM MES, pH 7.0, and 10 mM MgCl_2 at 50°C . Reactions were quenched, products separated by denaturing gel electrophoresis, and quantitated with an AMBIS radioanalytical scanner. *b*, Observed rate constant (k_{obs}) determined from slopes of plots analogous to that in *a*, plotted against the concentration of ribozyme. Open and closed symbols are from independent experiments and the square is from a reaction with a different preparation of ribozyme. The initial slope of this line gives (k_{cat}/K_m) (apparent) = $180 \text{ M}^{-1} \text{ min}^{-1}$; the line is theoretical for this value of (k_{cat}/K_m) (apparent) and $K_m = 30 \text{ } \mu\text{M}$ (see text). The value of $K_m(\text{G}) = 1 \text{ mM}$ (data not shown) and the concentration of G of 0.8 mM were used to extrapolate (k_{cat}/K_m) (apparent) to the value with saturating G of $k_{\text{cat}}/K_m = 400 \text{ M}^{-1} \text{ min}^{-1}$ for the DNA substrate. This correction does not affect any of the conclusions derived from these data.

could be outside of the active site as the E-S complex has roughly the stability expected for a simple DNA-RNA hybrid (see above). The electron-withdrawing effect of the 2'-hydroxyl group and its ability to form an intramolecular hydrogen bond with the 3' oxygen atom, which make the oxanion of ribose a better leaving group than that of deoxyribose, could contribute to the faster reaction of the RNA substrate. But it seems likely that metal ion coordination or general acid catalysis would be required to achieve the large amount of catalysis observed in this reaction, and that such catalysis would reduce the charge development on the 3' oxygen atom, thereby lowering the sensitivity of the reaction to the leaving-group ability of the ribose and deoxyribose.

As described above, the DNA substrate has both a lower affinity and slower rate of reaction after binding than the analogous RNA substrate. By contrast, an RNA substrate that forms a mismatch with the 5' exon-binding site at position -3 from the reaction site, binds less strongly than the matched substrate (Fig. 1), but reacts at essentially the same rate as the matched RNA substrate once bound (D.H. and T.R.C., manuscript submitted). Therefore, not all changes in substrate binding cause a change in the rate of the chemical step.

Even though the reaction of the DNA substrate is much slower than that of the RNA substrate, the DNA reaction still represents enormous catalysis relative to the solution reaction. The ribozyme is estimated to catalyse the DNA reaction by about 10^9 -fold. (This rate-enhancement is based on a k_c of 0.01 min^{-1} (Table 1) and an estimated rate constant for hydrolysis of dimethyl phosphate by water, k_w of $3 \times 10^{-12} \text{ min}^{-1}$. This value has been calculated from $k_{\text{OH}} = 8 \times 10^{-9} \text{ M}^{-1} \text{ min}^{-1}$ for reaction of dimethyl phosphate and hydroxide ion (attack at phosphorus; 50°C)^{23,24}, the linear free energy relationship $\beta_{\text{nuc}} = \delta(\log k)/\delta pK_{\text{nuc}} \sim 0.3$, for reaction of oxygen nucleophiles with a phosphate diester²⁵, and the concentration of water of 55 M : $k_w = 55 \text{ M} \times k_{\text{OH}} \times \exp\{\beta_{\text{nuc}} \times (pK_{\text{a}}^{\text{H}_2\text{O}} - pK_{\text{a}}^{\text{HO}^-})\}$.) Therefore, although the 2'-hydroxyl group seems to be involved in one catalytic strategy by the ribozyme, there are additional strategies operative with both DNA and RNA substrates. Similarly, individual protein enzymes seem to use several different catalytic strategies to achieve rate increases comparable to that obtained with the ribozyme^{26,27}.

No cleavage of dC₅ was observed in earlier work with a different ribozyme derived from the *Tetrahymena* IVS³. The weaker binding of dC₅ than d(CCCUCUA₅), and the preference for U over C at the position preceding the reaction site^{17,18} presumably contributed to the reason why no reaction was observed.

Implications and applications

Several group I IVSs are mobile genetic elements²⁸. Each of these has an open reading frame (ORF) encoding a protein that acts as a sequence-specific endonuclease. This endonuclease cleaves double-stranded DNA and initiates conversion of the gene from IVS⁻ to IVS⁺. The new finding of inherent DNA-cleavage activity in a group I IVS RNA indicates an extension of this idea: before the acquisition of an ORF, the RNA could have served as its own DNA endonuclease. Therefore the IVS could have initiated its mobility to an uninterrupted version of the same gene or its transposition to a new site, and could still do so in the case of group I IVSs, such as the *Tetrahymena* IVS, that do not contain an ORF. Considering that the IVS can use its own 3'-terminal G as an attacking group as an alternative to exogenous G (Fig. 2; ref. 3, and D. L. Robertson and G. F. Joyce, manuscript submitted), another version of this reaction would involve the insertion of the IVS RNA directly into a DNA genome by a two-step reaction analogous to the reverse of self-splicing (Fig. 2 and ref. 29). In either case, the DNA cleavage reaction that initiated insertion would necessarily involve the same sequence recognition as is required for RNA splicing; therefore, if the IVS inserted into the noncoding DNA strand, transcripts of the newly interrupted gene would undergo self-splicing at the RNA level, preventing gene inactivation. The slow reaction with DNA and the presumed requirement for single-stranded DNA would be expected to render such insertion events rare.

The finding of catalytic activity of RNA molecules rekindled speculation that an 'RNA world', in which RNA both provided catalysis and stored information, pre-dated life with protein catalysts^{30,31}. Our finding that an RNA catalyst can act on a DNA substrate indicates that an RNA world could have expanded to include DNA before the involvement of proteins⁷. We note that the *Tetrahymena* ribozyme has been selected to catalyse reactions of RNA substrates, and it is therefore possible that there are different RNA catalysts considerably better at catalysing reactions of DNA substrates (D. L. Robertson and G. F. Joyce, manuscript submitted).

It is conceivable that the DNA endonuclease reaction will have some practical application. The reaction is sequence-specific (Fig. 3), and site-directed mutagenesis of the 5' exon-binding site (Fig. 1) should allow cutting of a large variety of DNA substrates, as has been accomplished with the RNA endonuclease reaction^{32,33}. Lengthening the internal guide sequence to improve binding should be one way to enhance the rate of the DNA endonuclease reaction. □

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A sodium–potassium switch in the formation of four-stranded G4-DNA

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Single-stranded complex guanine-rich DNA sequences from chromosomal telomeres and elsewhere can associate to form stable parallel four-stranded structures termed G4-DNA by a process that is anomalously dependent on the particular alkali metal cation that is present. The anomaly, which is not found in the formation of G4-DNA by oligonucleotides containing short, single runs of three or more guanines, is caused by potassium cations excessively stabilizing fold-back intermediate structures, or pathway by-products.

GUANINE-rich sequences, often short runs of guanines in longer repeated sequences, occur in many chromosomal locations, such as in immunoglobulin switch regions¹, in telomeres², where they form overhanging 3' ends, in gene promoters^{3,4}, and in variable number of terminal repeat (VNTR⁵) regions. Under conditions of moderate-to-high sodium concentration, single-stranded DNAs from such regions aggregate to form four-stranded struc-

tures (G4-DNA)⁶ in which the strands run in parallel orientation and are linked by Hoogsteen-bonded guanine quartets. However, G-rich single-stranded DNAs can also associate to form antiparallel structures held together by guanine base pairs or quartets^{7–11}. Oligomers from telomeric sequences can form simple intramolecular fold-back structures at low salt concentrations, which migrate ahead of the input oligomer during electrophoresis⁷; oligonucleotides corresponding to the telomeres of *Oxytricha* and *Tetrahymena* can interact by combining two hairpin fold-back structures to give guanine quartet-linked 'dimer' structures^{8,9}; and stretches of 27–37 guanines in denatured plasmids can fold into four-stranded antiparallel structures^{10,11}.

Here we describe a novel electrophoretic assay which demonstrates that G4-DNA, including that formed by the telomeric sequences that also give antiparallel dimers, is indeed four-stranded. In studying the rate of formation of G4-DNA and the effects of ions on that rate, we find that complex sequences will form G4-DNA only in the presence of sodium and rubidium ions, but not in the presence of potassium ions. We show that this anomaly arises because potassium ions stabilize short G4-DNA structures so intensely that transient intermediates or by-products of the longer G4 structures are trapped.

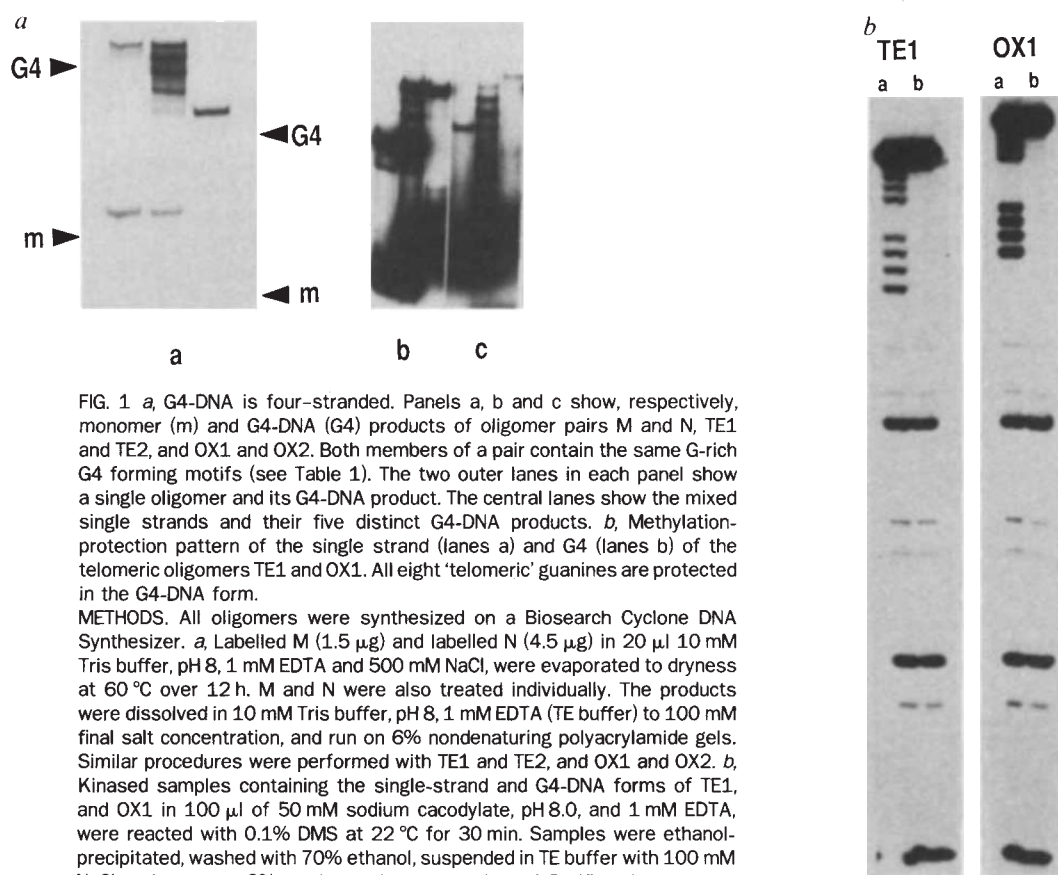


FIG. 1 *a*, G4-DNA is four-stranded. Panels *a*, *b* and *c* show, respectively, monomer (*m*) and G4-DNA (*G4*) products of oligomer pairs *M* and *N*, *TE1* and *TE2*, and *OX1* and *OX2*. Both members of a pair contain the same G-rich G4 forming motifs (see Table 1). The two outer lanes in each panel show a single oligomer and its G4-DNA product. The central lanes show the mixed single strands and their five distinct G4-DNA products. *b*, Methylation-protection pattern of the single strand (lanes *a*) and G4 (lanes *b*) of the telomeric oligomers *TE1* and *OX1*. All eight 'telomeric' guanines are protected in the G4-DNA form.

METHODS. All oligomers were synthesized on a Biosearch Cyclone DNA Synthesizer. *a*, Labelled *M* (1.5 μ g) and labelled *N* (4.5 μ g) in 20 μ l 10 mM Tris buffer, pH 8, 1 mM EDTA and 500 mM NaCl, were evaporated to dryness at 60 °C over 12 h. *M* and *N* were also treated individually. The products were dissolved in 10 mM Tris buffer, pH 8, 1 mM EDTA (TE buffer) to 100 mM final salt concentration, and run on 6% nondenaturing polyacrylamide gels. Similar procedures were performed with *TE1* and *TE2*, and *OX1* and *OX2*. *b*, Kinased samples containing the single-strand and G4-DNA forms of *TE1*, and *OX1* in 100 μ l of 50 mM sodium cacodylate, pH 8.0, and 1 mM EDTA, were reacted with 0.1% DMS at 22 °C for 30 min. Samples were ethanol-precipitated, washed with 70% ethanol, suspended in TE buffer with 100 mM NaCl, and run on an 8% nondenaturing preparative gel. Purified single-strand DNA and G4-DNA were treated with piperidine and run on a 20% sequencing gel.

TABLE 1 Oligomers

M (47mer):	5'-TAGTCCAGGCTGAGCAGGTACGGGGGAGCTGGGGTAGATGGAATGT
N (71mer):	5'-GGAATGTTAGGAGTCCAGGCTGAGCAGGTACGGGGGAGCTGGGGTAGATTGGAATGTAGGAGTCCAGACTG
OX1 (32mer):	5'-ACTGTCGTAATTGATATTTGGGGTTTTGGGG
OX2 (43mer):	5'-ACTGTCGTAATTGATACTGTCGCACTATTTGGGGTTTTGGGG
TE1 (28mer):	5'-ACTGTCGTAATTGATATTTGGGGTTGGGG
TE2 (39mer):	5'-ACTGTCGTAATTGATACTGTCGCACTATTTGGGGTTGGGG
P (49mer):	5'-GGGACCAGACCTAGCAGCTATGGGGGAGCTGGGGAAGGTGGGAATGTGA
Q (14mer):	5'-GGGGAGCTGGGGT
S (8mer):	5'-TTATTGGG
T (15mer):	5'-TTACTTGTATTGGG
U (12mer):	5'-TGATATTGGGGA
V (23mer):	5'-ACTGTCGTAATTGATATTTGGGGC
X (12mer):	5'-TGATATTGGGGC
Y (23mer):	5'-ACTGTCGTAATTGATATTTGGGGT

Stoichiometry and structure of G4-DNA

A DNA oligomer containing certain G-rich sequences in a longer sequence, when treated to form G4-DNA, yields a unique product of defined, slow electrophoretic mobility. Therefore, if two dissimilarly sized oligomers, A and B, containing the same G4-forming motif, were mixed and allowed to form G4-DNA, in principle, five distinct four-stranded products, corresponding to A_4 , A_3B , A_2B_2 , AB_3 and B_4 , should be resolvable on a nondenaturing gel. Indeed, with the pairs of oligomers (all oligomers are listed in Table 1) M and N, and TE1 and TE2, which end in a sequence that is a single-stranded 3' overhang in *Tetrahymena* telomeres¹²⁻¹⁴, and OX1 and OX2, containing the corresponding sequence from *Oxytricha* telomeres¹², the predicted five bands were produced (Fig. 1a), demonstrating directly the four-stranded nature of the product.

Figure 1b shows the results of methylation-protection experiments, demonstrating a characteristic total protection of the eight 'telomeric' guanines at the 3' ends of the oligomers TE1 and OX1 in the G4-DNA form.

Dependence on DNA concentration

Because G4-DNA is four-stranded one might expect its rate of formation to be quartic in DNA concentration, assuming a simple mechanism. To measure this rate, we incubated a series of samples of TE2 and OX1 at total DNA concentrations of 0.1, 0.3, 1.0, 3.0 and 10.0 $\mu\text{g } \mu\text{l}^{-1}$ ($\sim 8\text{--}800 \mu\text{M}$) of each oligomer but containing equal amounts of the corresponding radiolabelled oligomer in sealed glass capillaries at 60 °C. The rate of formation of G4 DNA was slow; yields monitored each day, from day 0 to day 5, increased linearly, demonstrating that the system was far from equilibrium. Figure 2 shows the electrophoresis of the products after 2 days (a), and a graph of the yield of G4 DNA versus the starting DNA concentration (b), giving a slope of two for each oligomer, indicating an unexpected quadratic dependence of G4-DNA formation. The second-order rate constant is $4.5 \times 10^{-4} \text{ M}^{-1} \text{ s}^{-1}$ at 60 °C in 1 M NaCl at pH 8.

Alkali metal ions and G4-DNA formation

RNA homopolymers poly(I) and poly(G) form parallel four-stranded structures¹⁵⁻¹⁹ in the presence of certain monovalent cations; even guanosine monophosphates (5' and 3'GMP) form gels or other ordered structures²⁰⁻²⁶ with quasi-quadruple-helical arrays. Different alkali metal ions have widely differing efficacies in promoting the formation of these structures, with K^+ being much more effective than Na^+ or Rb^+ , whereas Li^+ is ineffective in all cases, and Cs^+ ineffective for GMP. The K^+ complexes also have the greatest thermal stability. A crown ether-like size-selective binding of cations²⁷ in the central cavity of the G and I base quartets could explain this phenomenon.

To investigate the roles of ions in the formation of G4-DNA, we incubated the oligomers P, OX1 and TE2 with the five alkali metal ions. Figure 3a shows the results for P and TE2. For all three oligomers, only Na^+ and Rb^+ favoured G4-DNA

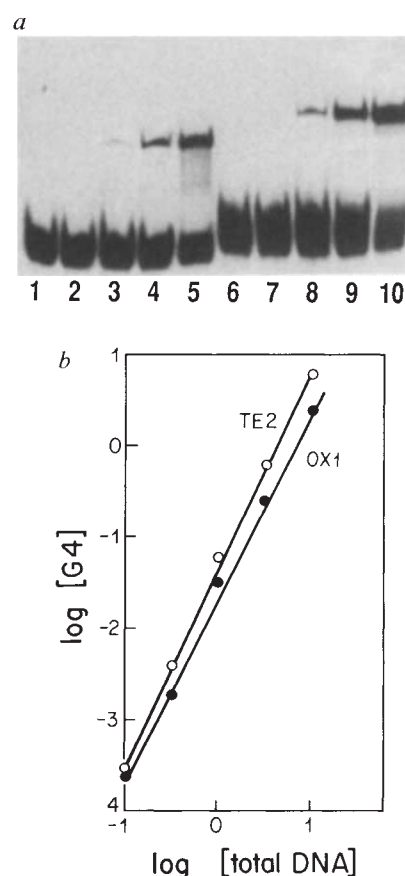


FIG. 2 a, Dependence of rate of G4-DNA formation on DNA concentration. Lanes 1–5 show G4-DNA (upper band) formation by the oligomer OX1; starting DNA concentrations were 0.1, 0.3, 1.0, 3.0 and 10.0 $\mu\text{g } \mu\text{l}^{-1}$ (8–800 μM), respectively. Lanes 6–10 show the same concentration series for TE2 (one absorbance at 260 nm (A_{260}) unit for all oligomers assumed to correspond to 40 μg). b, Plot of the concentration of G4-DNA [G4] against total DNA concentration [total DNA] for the oligomers TE2 and OX1. The plot shows a quadratic dependence on concentration.

METHODS. Oligomers TE2 and OX1, denatured at 100 °C for 4 min in TE buffer, pH 8.0, were suspended in TE buffer, pH 8.0, with 1 M NaCl in 10- μl samples each containing an equal amount of denatured labelled oligomer and sufficient denatured unlabelled oligomer to give final DNA concentrations of 0.1, 0.3, 1.0, 3.0 and 10.0 $\mu\text{g } \mu\text{l}^{-1}$ DNA, respectively. Samples were sealed in 25- μl glass capillaries and incubated at 60 °C for 2 days, then diluted fourfold with TE buffer (G4-DNA, once formed, is stable to this dilution) and run on an 8% nondenaturing gel at room temperature and in 50 mM TBE. The gel was dried on DEAE paper (Bio-Rad; Whatman). Densitometry was performed on different exposures of this gel, strictly within the linear response of film. Raw G4/(G4 plus single strand) data were scaled to concentrations, necessary because 1 unit labelled DNA in the different samples represented 1, 3, 10, 30 and 100 units of total DNA, respectively.

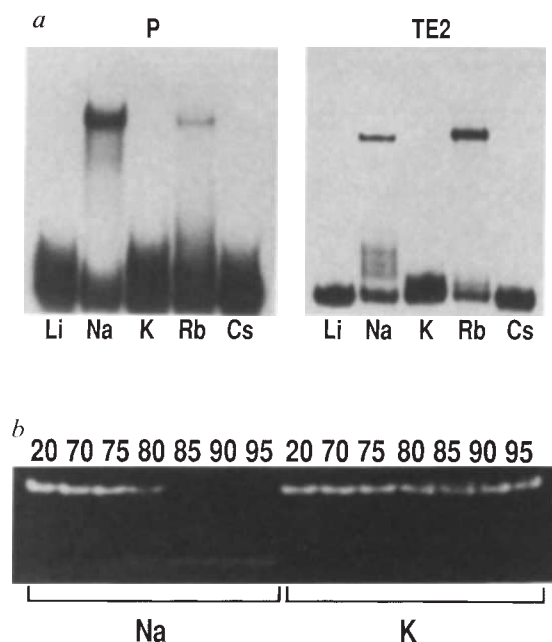


FIG. 3 *a*, Influence of different alkali cations on G4-DNA formation. G4-DNA appears in sodium and rubidium solutions, but not in potassium solutions. *b*, Relative thermal stability of the G4-DNA form (upper band) of Q in sodium and potassium buffers. In potassium, the complex is stable at temperatures of at least 20 °C higher.

METHODS. *a*, P and TE2 were suspended at $3 \mu\text{g } \mu\text{l}^{-1}$ in 20 μl each of TE buffer, pH 8.0 with 1 M XCl (where X represents Li, Na, K, Rb or Cs). Samples were sealed in glass capillaries and incubated at 60 °C for 24 h. Recovered samples were diluted fourfold and run on a 6% nondenaturing gel (for P) or an 8% gel (for TE2). *b*, Aliquots (15 μl) of the G4-DNA form of Q, at $1 \mu\text{g } \mu\text{l}^{-1}$ in 25 mM NaPi (40 mM Na⁺), pH 7.0, 0.5 mM EDTA, and 125 mM of either NaCl or KCl, were incubated for 8 min each in a Perkin Elmer Cetus thermal cycler set at different temperatures. Samples were then chilled on ice and run in an 8% gel in 50 mM TBE buffer at 4 °C. The gel was stained with ethidium bromide.

formation; K⁺ was ineffective (tested at concentrations between 5 mM to 1 M).

Because G4-DNA did not form in K⁺ solutions, we tested whether K⁺ added subsequently would stabilize or destabilize the G4-DNA complex. We followed the hyperchromicity of the G4-DNA form of the short oligomer Q as a function of temperature in either 165 mM Na⁺ or 125 mM K⁺/40 mM Na⁺. There was a sharp change in the absorbance at 255 nm of the Na⁺ sample at >80 °C, but no comparable change in the absorbance of the potassium sample (data not shown). To verify that the transition reflected melting of the G4-DNA, we monitored it by gel electrophoresis. We incubated samples at different temperatures for 8 min, chilled them on ice, and ran them on a nondenaturing gel at 4 °C. Figure 3*b* shows the results for the Na⁺ and the Na⁺/K⁺ buffers described above. There is a clear melting of the G4 DNA in the sodium sample at >80 °C, whereas in the potassium sample there is no appreciable breakdown, even at 95 °C. Comparison of the alkali cations yielded the following order for their efficiency at stabilizing G4-DNA: K > Rb > Na > Li or Cs.

Sodium-potassium switch for G4-DNA formation

We examined the rate of G4-DNA formation by P, TE1, TE2 and OX1 in a series of buffers with mixtures of alkali metal chlorides. In lithium and sodium mixtures there was little change in the kinetics of G4-DNA formation on replacing half of the sodium with lithium at a 1 M concentration. Sodium-potassium mixtures ranging from 1 M to 150 mM, at 37 °C and 60 °C,

however, gave very different results. There are two phases of G4-DNA formation (Fig. 4, *a* and *b*): in low molar ratios of potassium, there is a progressive increase in the rate of G4-DNA formation (up to an order of magnitude higher than with sodium alone), whereas at higher potassium ratios, G4-DNA formation is inhibited. (At 1 M total salt, however, P showed only a consistent decline of G4-DNA formation with increasing potassium concentration, unlike TE1 and OX1, which still showed the initial range of stimulation; data not shown.)

All oligomers, especially the telomeric ones, gave product bands other than the G4-DNA band that migrated just above the single-strand band; the distribution of these bands differed between the sodium and potassium experiments. Figure 4*a* shows that a well-defined band (labelled 'K') is the principal product of the treatment of P with potassium. To probe the structure of the K product, we treated it with dimethylsulphate and ran the methylated and piperidine-treated G4-DNA and K products of P side-by-side on a sequencing gel (Fig. 5*a*). The methylation protection patterns of the K and G4-DNA products are different. Whereas the G4-DNA form shows the characteristic total protection of guanines across the 21-base G-rich stretch, the K product shows protection only in two four-guanine stretches (four protected in one, and two in the other). A guanine bordering one of these motifs is methylation-enhanced. Product K is probably a dimer; it migrates more slowly than the monomer rather than faster as might a single fold-back structure. Figure 5*b* shows the structures most consistent with the methylation pattern of product K: antiparallel fold-back structures consisting of two oligomers and containing guanine quartets, similar to the dimer products postulated by others^{8,9}. The other 'minor' bands described above are likely to comprise similar fold-back

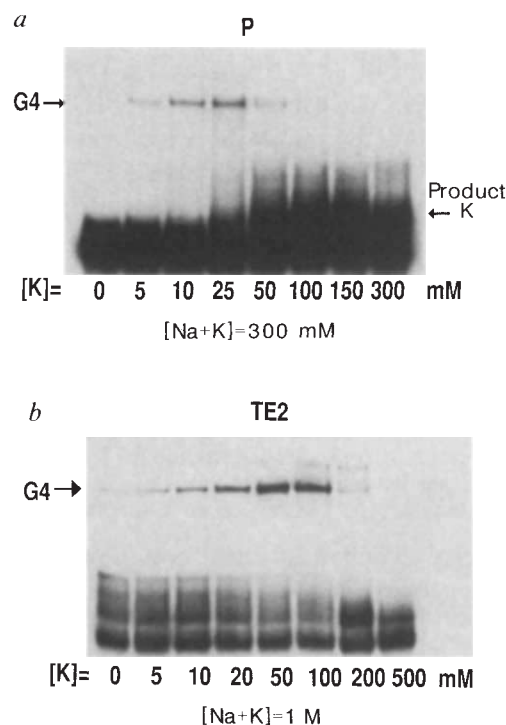


FIG. 4 Rate of formation of G4-DNA in constant ionic-strength buffers containing varying proportions of sodium and potassium. Increasing potassium-ion concentration first stimulates, then blocks, G4-DNA formation. [K], concentration of K⁺; [Na+K], total Na⁺ and K⁺ concentration. METHODS. Oligomer P, and TE2, heat-denatured as in Fig. 2, were divided into 10- μl samples of $2 \mu\text{g } \mu\text{l}^{-1}$ each, in buffers containing TE, pH 8.0, with 300 mM or 1 M (NaCl plus KCl). Samples were sealed in glass capillaries and incubated at 60 °C for 60 h. Recovered samples were loaded on 6% gels, which were dried on DEAE paper.

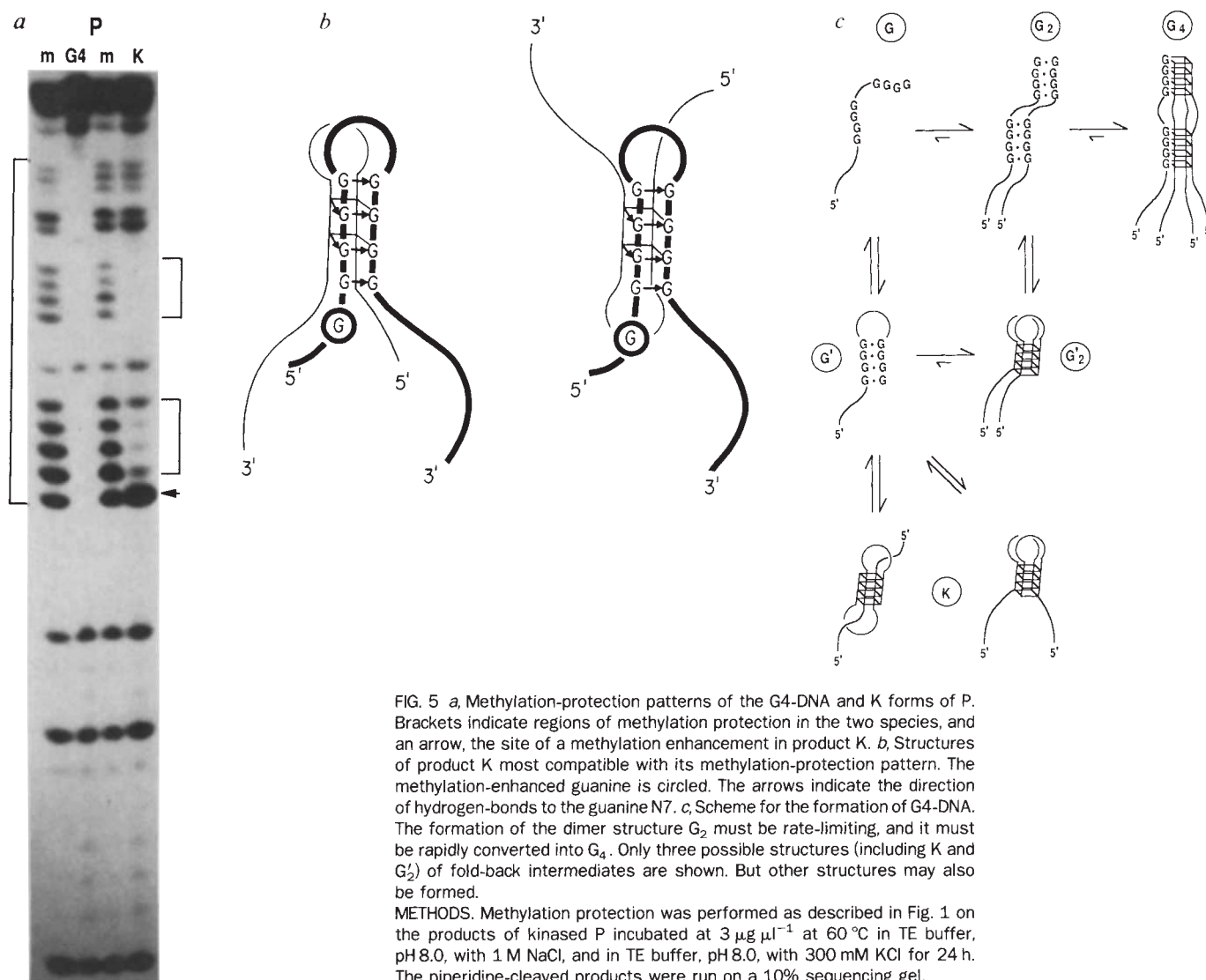


FIG. 5 *a*, Methylation-protection patterns of the G4-DNA and K forms of P. Brackets indicate regions of methylation protection in the two species, and an arrow, the site of a methylation enhancement in product K. *b*, Structures of product K most compatible with its methylation-protection pattern. The methylation-enhanced guanine is circled. The arrows indicate the direction of hydrogen-bonds to the guanine N7. *c*, Scheme for the formation of G4-DNA. The formation of the dimer structure G_2 must be rate-limiting, and it must be rapidly converted into G_4 . Only three possible structures (including K and G'_2) of fold-back intermediates are shown. But other structures may also be formed.

METHODS. Methylation protection was performed as described in Fig. 1 on the products of kinased P incubated at $3 \mu\text{g } \mu\text{l}^{-1}$ at 60°C in TE buffer, pH 8.0, with 1 M NaCl, and in TE buffer, pH 8.0, with 300 mM KCl for 24 h. The piperidine-cleaved products were run on a 10% sequencing gel.

structures, with different relative geometries of the two constituent oligomer molecules.

A model for G4-DNA formation

Figure 5c shows a model consistent with the experimental data on G4-DNA formation. A direct route is to first form dimers, G_2 , which interact to form G_4 . An alternative route to G_2 is for folded-back single strands to dimerize to G'_2 , which could then unfold. The folded monomers could also dimerize to give antiparallel structures, K. The guanine base-quartets in all these dimers require coordinated cations for stabilization.

The quadratic dependence on the forward rate indicates that the formation of a critical dimer intermediate is the rate-limiting step. This indicates that the two reverse rates for the decay of G_2 and the decay of G_4 are very small, and that G_2 is depleted by conversion to G_4 and G'_2 as fast as it is made. Both the stabilization by potassium of G'_2 and K (and possibly also G'), and the inability of G'_2 to unfold to G_2 , would explain the block of G_4 formation in potassium solutions.

Single G-motif oligomers and G4-DNA formation

The interpretation of the potassium effect as over-stabilization of dimer fold-back structures leads to the predictions that (1) a short run of guanine residues in an oligomer should be sufficient to associate intermolecularly to four-stranded structures, whether of parallel or antiparallel orientation, (2) these

structures should form with both sodium and potassium, and (3) the potassium complexes, once formed, should be more stable than the sodium complexes. We treated the sets of oligomers S and T, U and V, and X and Y, containing three, four and five contiguous guanines respectively, for the five-band experiment. Figure 6a shows the products from X and Y at 200 mM NaCl and 200 mM KCl at 37°C . Four-stranded products were formed by these simple oligomers, with both NaCl and KCl. U and V gave similar results; with S and T, 200 mM K^+ was effective at 22°C , whereas the five bands appeared only weakly with 1 M Na^+ .

Figure 6b shows the melting profile of the G4-DNA product of U in 50 mM NaCl and in 50 mM KCl. The sodium complex melts at about 40°C , whereas the potassium sample is fully stable at 70°C , melting, in fact, at above 90°C (data not shown).

Discussion

Guanine-rich DNA sequences can assume a range of higher-order structures. Apart from G4-DNA⁶, there are various antiparallel fold-back structures⁷⁻¹¹. The methylation protection experiments with oligomers with asymmetric guanine motifs⁶, and also the precedent of poly(I) and poly(G) fibre-diffraction data^{15,16}, indicate that the four strands in G4-DNA run in parallel orientation. But, at least in intramolecular complexes, antiparallel guanine quartets form. Antiparallel structures must

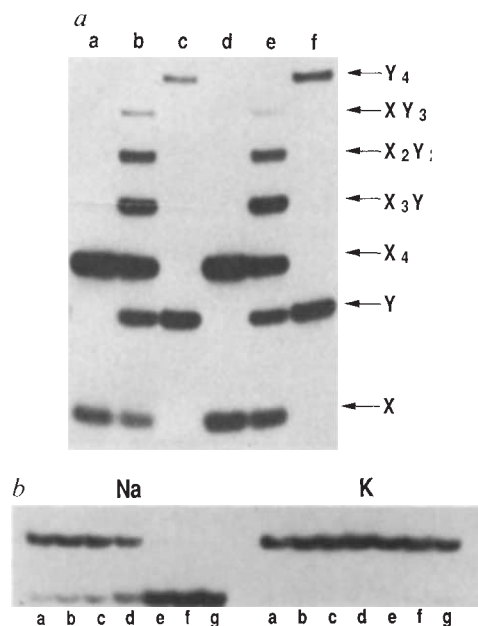


FIG. 6 Single guanine-motif oligomers form G4-DNA with both sodium and potassium. *a*, G4-DNA products of the oligomers X and Y formed in Na^+ and in K^+ solutions. Lanes a–c, results with 200 mM NaCl; lanes d–f, results with 200 mM KCl. Patterns show the presence of five product bands arising from the four-stranded nature of the complex. *b*, Relative thermal stabilities of U_4 in TE buffer and 50 mM NaCl or KCl. Lanes: a, 0 °C; b, 20 °C; c, 30 °C; d, 40 °C; e, 50 °C; f, 60 °C; g, 70 °C.

METHODS. *a*, Oligomers were heat-denatured as in Fig. 2, suspended in 10- μl samples containing $1.6 \mu\text{g} \mu\text{l}^{-1}$ X, $1.6 \mu\text{g} \mu\text{l}^{-1}$ X and $2.25 \mu\text{g} \mu\text{l}^{-1}$ Y, and $2.25 \mu\text{g} \mu\text{l}^{-1}$ Y in TE buffer, pH 8.0 with 200 mM NaCl or KCl. Samples were incubated 12 h at 37 °C and run on an 8% gel in 50 mM TBE buffer at room temperature. (Gels for oligomers S, T, U and V were run at 4 °C in 50 mM TBE buffer + 5 mM KCl.) *b*, U treated to give G4 DNA with TE buffer and 200 mM NaCl, and, separately, KCl, was diluted to concentrations of TE, pH 8.0, and 50 mM NaCl or KCl, and divided into 15- μl aliquots. These were heat-treated and analysed as in Fig. 3b.

contain guanine residues in both the *syn* and *anti* conformations, which has been verified by NMR⁷. The parallel quadruple strands of poly (rG) and poly (rI) have all their bases in the *anti* conformation¹⁵, which could be more stable. (Preliminary results on oligomers S and T, containing three contiguous G residues each, show that their mixture forms small quantities of products of substantially lower mobility than the G4-DNA products. These could represent less stable antiparallel four-stranded products.) There seems to be considerable flexibility of nucleotide conformation in the guanine quartets. The G quartets in the G_2 complex, which would have a *syn-syn-anti-anti* conformation, would give rise to G_2 with one G motif of entirely *syn* conformation and the other G motif of entirely *anti* conformation. The resultant G4 DNA could maintain this arrangement, or, the *syn* motif in G_2 could relax to an *anti* conformation before forming G4-DNA.

An important result of our study is that as few as three contiguous guanines in a larger sequence can readily form G4-DNA in 0.2–0.5 M sodium or potassium. But the G4-DNA-forming property of sequences with several G motifs is strongly dependent on the balance of Na^+ and K^+ , which have, at different ratios, both synergistic and antagonistic effects. Generally, our experiments have focused on stable products (that is, G4-DNA) from G-rich oligonucleotides. Other investigators⁹ have found that fold-back 'dimer' products, which could be either intermediates or by-products of G4-DNA formation, are unstable (unless stabilized by K^+) under the gel conditions that we used, that is, 50 mM TBE buffer and room temperature. The

telomeric oligomers that we used, however, formed a range of 'dimeric' products (Fig. 4b), which differed between sodium and potassium conditions. The persistence of these products could be due to the loading of large amounts of DNA (~10 μg per lane) in our gels.

Homologous replicated chromosomes at meiosis, or elsewhere in the cell cycle, may use transient G4-DNA structures to achieve a four-fold recognition of 'self', before synapsis and genetic exchange⁶. Chromosomal telomeres, which have a single-stranded G-rich protruding tail, may form G4-DNA as an initiating step to such chromosomal pairing⁶. Here we further demonstrate the versatility of guanine-rich single strands. Telomeric single-strands in the cell may occur in several alternative structures: a strictly folded-back conformation, for recognition by the telomerase enzyme⁷; a twofold intermolecular or inter-chromatid association similar to the K structures of Fig. 5b; or a fourfold association, as in G4-DNA. The 'default' conformational state of the two-motif telomere ends could be a simple fold-back structure in the high-potassium cellular environment, but the cell could easily generate G4-DNA by blocking one of the two G motifs with a protein, whereupon the other motif would readily form quadruple strands.

Overall, we expect sequences with four or more separated runs of guanines to produce four-stranded intramolecular fold-back structures, sequences with two runs to produce dimer fold-back structures, and sequences with single runs to produce G4-DNA. All of these structures can ultimately be converted to four-stranded parallel structures. □

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Genesis of pulsars in globular clusters

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THE birth-rate of radio pulsars in globular clusters (GCs) is thought to be a factor of 10 to 100 times greater than that of their candidate progenitors, low-mass X-ray binaries (LMXBs) of canonical lifetime 10^8 to 10^9 yr. This apparent discrepancy^{1,2} would be removed either if there were more rapid accretion of mass onto the primary in the late stages of LMXB evolution (as suggested by the self-excited wind model³), or if there occurred accretion-induced collapse of white dwarfs in binaries with wide orbits^{4,5}. But the available data (Table 1) indicate that GC pulsars cannot be formed by either of these mechanisms alone. Here we suggest that both processes may be necessary to some extent, the corollary of which is that GC pulsars may have at least two distinct classes of progenitors. Many solitary and binary pulsars with short orbital periods may have descended from rapidly evolving LMXBs, whereas others, especially those with long periods, may have formed from binaries that undergo nuclear evolution of the secondary and eventual accretion-induced collapse of the white-dwarf primary.

There are three pertinent aspects to the problem of pulsar evolution in globular clusters: (1) the birth-rates of binary and recycled pulsars relative to that of LMXBs; (2) the abundance of LMXBs; and (3) the orbital period distribution of the known radio pulsars. We discuss these aspects in turn.

There are $N_x = 10$ neutron-star X-ray binaries (NSXBs) in GCs, of which nine exhibit X-ray bursts. Evolutionary models proposed for those sources give average rates of mass transfer onto neutron stars from stripped giants in close binaries^{6,7} ranging from $10^{-9} M_\odot \text{ yr}^{-1}$ to $10^{-8} M_\odot \text{ yr}^{-1}$, with typical rates closer to the lower value. Such rates correspond to X-ray lifetimes τ_x of a few times 10^8 yr. These timescales are not inconsistent with the observed luminosities of GC NSXBs, but they pose a problem^{1,2} for the birth-rate of NSXBs in relation to that of the pulsars. Kulkarni *et al.*² estimate the birthrate of GC binary pulsars to be $B_p \approx 10^{-6} \text{ yr}^{-1}$; comparison of this number with the NSXB birthrate N_x/τ_x yields the discrepancy mentioned above. (Recently, studies of the integrated radio luminosity of clusters Ter 5 and NGC6440 led Fruchter and Goss⁸ to the conclusion that B_p may be lower by a factor of ~ 2 to 6.) A suggestion has been made⁹ to ameliorate the problem in the case of LMXB systems appearing in the disk (where the discrepancy is even more acute) by decreasing their X-ray lifetimes to $\sim 10^7$ yr. Models^{10,11} for the eclipsing binary PSR1957+20 and Cygnus X-3, involving self-sustained wind³ from a companion star of very small mass ($\leq 0.1 M_\odot$) excited by a neutron-star primary, do indeed involve a strong mass-transfer phase, possibly decreasing the LMXB lifetime to such a value. A similarly decreased lifetime in the GC LMXBs would make the birth-rates of the progenitor-progeny systems nearly equal^{1,2}. A simple shortening of the X-ray active lifetime of LMXBs in a GC would solve the birth-rate problem, but it may be inconsistent with the observed number of GC NSXBs.

Only 10^{-4} of the galactic mass is contained in GCs, yet about a tenth of all galactic NSXBs are located there. This overabundance of NSXBs in GCs¹² has led to suggestions¹³ that they are formed by the tidal capture of a cluster star. The number, N_x , of NSXBs currently observable in GCs is related to the number, n_b^* , of neutron-star binaries that have formed in each GC core over its lifetime τ_{GC} ($\sim 10^{10}$ yr) and have undergone a phase of steady X-ray emission for τ_x yr: $N_x = \sum (\tau_x/\tau_{GC}) n_b^*$, where the sum extends over all GCs.

The computed rate¹⁴ of tidal captures over a GC lifetime

indicates that $\sim 3\%$ of the neutron stars in the core will undergo a tidal capture if the number density of the target main-sequence stars is $\sim 10^4 \text{ pc}^{-3}$ in the GC core. Many GCs, however, may have cores that have collapsed already¹⁵, in which stellar densities are higher; as many as 20% of the GCs whose cores are unresolved¹⁶ may have undergone collapse. In these, possibly up to 50% of the primordial neutron stars could have captured a companion⁵. Hence, although the actual number of GCs is nearly 150, the sum in the expression for N_x should be¹⁷ dominated by about a dozen GCs that produce, on average, a large n_b^* , perhaps several tens. Assuming that the X-ray lifetime is similar for all sources, the expression can be inverted to give

$$\frac{N_{GC} \langle n_b^* \rangle}{10^3} \sim \frac{10^8 \text{ yr}}{\tau_x} \times \frac{N_x}{10} \quad (1)$$

Here N_{GC} is the number of GCs, and the angular brackets signify an average over all GCs. If the left-hand side were close to unity, the X-ray lifetime would be $\sim 10^8$ yr. An analysis of the clusters M15 and 47Tuc has led Bailyn and Grindlay⁵ to estimate the total number of LMXBs as ~ 200 , corresponding to lifetimes of 5×10^8 yr.

As N_x is well determined from observations, the lifetime of NSXBs can be decreased only if the number of neutron-star binaries formed by tidal capture is found to be correspondingly larger (equation (1)). That would require a larger number of neutron stars to be retained in the GC core over its lifetime, which in turn requires a less steep initial mass function (IMF) of the GCs. Alternatively, the higher capture efficiency of the neutron stars in a few core-collapsed clusters having large central density could contribute a larger number of neutron-star binaries. In addition, the total number of binaries formed in a globular cluster is affected by mass-segregation effects¹⁸ in core-collapsed clusters (due to which the number of captures by massive stars—including neutron stars—is enhanced), as well as by velocity weighting of the number of neutron stars retained after formation in the globular-cluster core. Both of these effects can possibly increase estimates of the total number of LMXBs formed in GCs. The estimates will be amenable to better theoretical analysis when further observational data of a quality comparable to that of Omega-Cen and 47Tuc become available for a large number of clusters.

There is a possibility that the LMXB progenitors of GC pulsars form a population distinct from the observed NSXBs. If a small fraction (10%, say) of LMXBs were long-lived ($\tau_x \approx 10^9$ yr) whereas the remainder had a shorter lifetime (say, 10^7 yr), $\sim 90\%$ of NSXBs active at present would then be of the long-lived variety, whereas $\sim 90\%$ of 'recycled' pulsars would derive from the more numerous short-lived LMXBs. In this way, the high accretion rate necessary for 'accelerated' LMXB evolution can be reconciled with the rather low accretion rates for the GC NSXBs actually observed (rates of 10^{-9} to $10^{-8} M_\odot \text{ yr}^{-1}$ are inferred, under certain assumptions, from their low luminosity and from the presence of X-ray bursts).

The currently observed distribution of binary/recycled pulsar orbital periods poses perhaps the most serious problems for the pulsar evolutionary scenarios that involve only LMXBs. Collision and tidal capture of a neutron star in a GC core is most frequent for main-sequence stars. The maximum orbital period P_i following capture (after orbital circularization with constant angular momentum), when the target star has approximately the cluster turn-off mass (below which it is on the main sequence), is less than a day. To attain a longer orbital period, the binary has to evolve according to the scenario of Webbink *et al.*⁷: the secondary has to be more massive than the cluster turn-off mass if there is to be substantial evolution within a GC lifetime. But if the binary has an initial period $P_i \approx 1$ day, it expands after transfer of $\sim 0.6 M_\odot$ to a final orbital period of only ~ 10 days. Therefore, long-period systems such as 1620+26 and 1310+18 (whose periods are an order of magnitude larger than 10 days) are not easily explained on the basis of the capture

TABLE 1 Globular-cluster pulsars

Pulsar	Reference	Cluster	P_{spin} (ms)	P_{orb} (d)	Eccentricity	$M_c/M_\odot$
1821-24*	27	M28	3.05	—	—	—
1516+02A*	28	M5	5.5	—	—	—
1639+36*	29	M13	10	—	—	—
0021-72C†	30	47Tuc‡	5.76	†	?	?
2127+11C	31,32	M15	30	0.335	0.67	>0.95
1516+02B‡	28	M5	7.9	~1	large?	?
0021-72A	33,34	47Tuc‡	4.48	0.022	0.32	4×10^{-3} or 0.8
0021-72B	35	47Tuc‡	6.13	7-95	?	0.2-0.4
1746-20*	36	NGC6440	288.6	—	—	—
2127+11B*	37	M15	56	—	—	—
2127+11A*	38	M15	110.66	—	—	—
1620-26	39,40	M4	11.08	195	0.025	0.3-0.4
1310+18	32,41	M53	33	~270	?	?

* Solitary pulsar.

† Solitary or very wide binary.

‡ $v_{\text{orb}} > 20 \text{ km s}^{-1}$.§ The dispersion measure reported in refs 30 and 38 ($D = 65 \text{ pc cm}^{-3}$) for 0021-72A and B is incompatible with that of ref. 32 for 0021-72C ($D = 25 \pm 2 \text{ pc cm}^{-3}$). Clearly, not all three of the reported 0021-72 pulsars are really present in 47Tuc.

and subsequent nuclear evolution of a main-sequence dwarf by a neutron star^{1,4}.

Furthermore, consider the assumption that all low-mass binary pulsars (including solitary ones) in GCs are the product of LMXB evolution, and that the short-orbital-period ($P_{\text{orb}} < 1$ day or less) systems are the product of high mass transfer induced by a self-excited wind whereas the longer- P_{orb} systems are products of LMXBs that have mass transfer induced by the nuclear evolution of the secondary on a timescale τ_m of⁷

$$\tau_m = 1.1 \times 10^9 P_i^{-1} \text{ yr} \quad (2)$$

(here P_i is in days). Under this assumption, the birth-rate of the long- P_{orb} radio pulsars would be ten to a hundred times smaller than that of the short- P_{orb} pulsar systems descended from X-ray binaries with a mass-transfer timescale of $\sim 10^7$ yr. But this is clearly not the case—the numbers of observed long- and short- P_{orb} radio pulsars are roughly similar (Table 1) and there are no obvious selection effects that strongly favour one over the other. (It is true that high acceleration in very-short-period binaries, say for $P_{\text{orb}} < 1$ h, can cause such systems to be under-represented in pulsar surveys, but Kulkarni *et al.*² estimate from data on M15 and M3 that this selection effect caused no more than two out of three such pulsars to be missed ($\beta \approx 2$); Fruchter and Goss⁸ estimate that $\beta \approx 0$, that is, that no pulsars were missed because of this selection effect.)

Although the capture of a red giant by a neutron star can in principle give the right post-capture (initial) orbital period for the binary to evolve to required long periods in the case of some GC pulsars, the number of such systems formed would again be small, because of the paucity both of red giants and of their would-be partners, neutron stars. Instead, the capture of a red giant by a massive white dwarf and the subsequent accretion-induced collapse (AIC) to a neutron star in a wide orbit seems statistically more probable, because more white dwarfs than neutron stars are retained in the GC core. In this AIC scenario⁵, the amount of mass lost to the white dwarf during formation of the neutron star is postulated to be small, such that the binary is not disrupted but nevertheless undergoes a small orbit expansion so that the companion no longer fills the Roche lobe. At that stage, such long- P_{orb} systems would not become NSXBs and could be detected only through the pulsar's radio emission.

All the known pulsars in GCs are millisecond pulsars (we assume that those with longer periods have been spun down). Any pulsars formed in supernova explosions of primordial massive cluster stars would have crossed the death line¹⁹ (turned off in the radio) a long time ago. The GC pulsars then either must have been formed more recently in accretion-induced collapse of massive white dwarfs⁵ (where, it has been suggested²⁰, the magnetic field of the newly born pulsar could have lower values) or they must be 'recycled' neutron stars whose magnetic field

has decayed to low values and which have been spun up by accretion from a binary companion²¹. There are two means by which isolated pulsars may be created. In the case of short-period binaries, the companion may have been evaporated^{10,22}. This is thought to be the more common route, but a very small proportion of solitary pulsars could instead have been liberated by collisional disruption²³; 'ionization' rates of binaries computed by Rappaport *et al.*²⁴ indicate that this could indeed have happened for the solitary pulsars in M15 (but not for that in M28). Out of thirteen GC pulsars reported so far, six or seven are solitary, three are in short-period binaries and three or four are in long-period binaries. We believe that the first three or four pulsars listed in Table 1 are descendants of short-lived LMXBs, whereas the last three to five had long-orbital-period progenitors. The status of the middle five is uncertain at present. The data in Table 1 therefore suggest that the number of long- and short-orbital-period progenitor binaries are comparable.

Accretion-induced collapse is envisaged to happen under rather special circumstances. In particular, the white dwarf has to be sufficiently massive initially that it can grow to the Chandrasekhar limit and collapse to form a neutron star. Furthermore, the mass-transfer rate needs to be high enough that the white-dwarf mass increases, rather than decreasing in nova flash ejections. Mass-transfer rates higher than $4 \times 10^{-8} M_\odot \text{ yr}^{-1}$ are required for AIC²⁵. Such transfer rates are possible in systems fed by red giants²⁶ and require long orbital periods, $P_i \geq 45$ days, leading to a final $P_{\text{orb}} \geq 200$ days. AIC can also take place in a common-envelope phase, where the mass-transfer rates can be large.

Only a small number of the shorter-orbital-period binaries containing white dwarfs can undergo accretion-induced collapse, because otherwise the ratio of the number of long- P_{orb} pulsars to short- P_{orb} pulsars would be small. Indeed, the fraction of all tidal captures that involve a giant is $\sim 7\%$ (ref. 17); mass segregation can increase that fraction to 30% (ref. 18). Thus, AIC of white dwarfs in short-orbital-period systems containing main-sequence companions must be very inefficient. Bailyn and Grindlay⁵ argue that the number of massive white dwarfs that manage to remain in GC cores and form binaries through tidal capture is 25 to 50 times the number of binaries formed by neutron stars. The number of binaries comprising a white dwarf that has captured a giant in a GC core is then roughly similar ($\sim 0.07 \times 25$) to the number of those comprising neutron stars with main-sequence companions (mass segregation increases the first factor in this product, and the requirement that the orbit be wide for AIC to be possible reduces the second factor). Moreover, the lifetimes of LMXBs undergoing self-excited mass transfer are comparable to the maximum lifetime allowed for AIC, so the birth-rate of the long- P_{orb} systems by AIC should be comparable to the birth-rate of short- P_{orb} systems formed

through 'accelerated' evolution of neutron-star binaries. Thus, in this scenario there should be roughly the same number of long- P_{orb} binary pulsars as short- P_{orb} (or single) systems, which is in fact the case.

Thus the orbital-period distribution of pulsars suggests that binary/recycled pulsars have two sets of progenitors: short-lived LMXBs (such as those undergoing mass transfer through self-excited winds) form one class, and the other contains white dwarfs (about to form a neutron star through AIC) undergoing mass transfer from nuclear-evolved secondaries in wider binaries, which are generally invisible in the X-ray band except in deep surveys. The combination of a larger number of white dwarfs than neutron stars and a smaller number of nuclear-evolved subgiant or giant stars relative to main-sequence stars would make the number of the progeny pulsars from AIC roughly equal to those arising from LMXBs evolving rapidly through self-excited winds.

Note added in proof: Lyne *et al.*⁴² have reported the discovery of an eclipsing binary pulsar in the cluster Terzan 5, with $P_{\text{spin}} = 11.56$ ms, $P_{\text{orb}} = 1.7$ h, $e < 0.01$, $M_c = 0.1 M_{\odot}$. The eclipse length is almost half the orbital period and the pulsar is two core radii from the cluster centre. Similarity of the system to PSR1957+20 may support a common evolutionary origin of the two binaries, and may support the idea that GC binary pulsars with short orbital periods are formed in a short-lived LMXB phase of evolution driven by self-excited wind^{3,10}. On the other hand, this particular system may have formed in a (relatively recent) accretion-induced collapse of a white dwarf which had collided head-on with its giant containing a degenerate core and spiralled in from an initial orbital period of approximately a day. □

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Elliptical-like light profiles in infrared images of merging spiral galaxies

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THEORETICAL studies indicate that in collisions between disk galaxies, dynamical friction and subsequent relaxation will eventually produce a mass distribution indistinguishable from that of an elliptical galaxy. However, there are also theoretical and observational arguments against this process^{1,2}. Schweizer's pioneering paper³, which showed that the V-light profile of the merging galaxy NGC7252 is indeed well fitted by the $r^{1/4}$ de Vaucouleurs law, remains the only observational evidence in support of this idea. In most candidate mergers the optical structure is complicated because of emission from star-forming regions and obscuration by dust lanes, and there is no overall resemblance to ellipticals. In the near-infrared, extinction and the effects of hot young stars are much reduced. Accordingly we have obtained K images of six galaxies which are believed to be mergers of disk galaxies. Two of the six, NGC2623 and Arp220, appear to obey the $r^{1/4}$ law to the limits of our images, offering new support for the idea that mergers of spirals can evolve into elliptical-like objects.

The images were obtained at the UK Infrared Telescope (UKIRT) on the night of 30 April 1987 using the infrared camera IRCAM^{4,5} in its highest-resolution mode (0.62 arcsec per pixel), with a standard K (2.2 μm) filter. Six candidate mergers with high infrared luminosities selected from the sample of Joseph and Wright⁶ were observed. Images of the galaxies were taken alternately with nearby regions of blank sky which were used to form the flat field. After dark current subtraction and flat fielding (removing pixel-to-pixel responsivity variations), the mean sky brightness, measured on the image frame, was subtracted. Here we mainly discuss the results for NGC2623 and Arp220. The final image of NGC2623 is the average of three independent images of 240-s exposure time, each shifted slightly relative to the previous one to remove the effects of bad pixels. For Arp220, four 120-s images were similarly co-added. Sky conditions were poor, so no standard stars were observed. The seeing averaged 2 arcsec at full width half maximum. The images were calibrated using K photometry of the nuclei obtained in an aperture of 5 arcsec (ref. 7). We derived light profiles from the reduced images by integrating within elliptical apertures of increasing size. The 'best fit' ellipticity and centre for the ellipse were found by fitting by eye to contours ~ 10 arcsec in diameter. In the following analysis the radii referred to are the geometric means of the semi-major and semi-minor axes of the ellipse. The main source of error in the derived profiles arises from the systematic uncertainty in the sky level. Within the fluctuations of the levels on the blank sky frames we find no systematic difference between the sky values derived from the edge of the image frame and those derived from blank sky. We have therefore used the standard deviation of the sky counts on the independent images to estimate the resulting uncertainty in the surface photometry.

Optical photographs of these galaxies (see ref. 6) show multiple nuclei, tidal tails and filaments. In particular, Sanders *et al.*⁸

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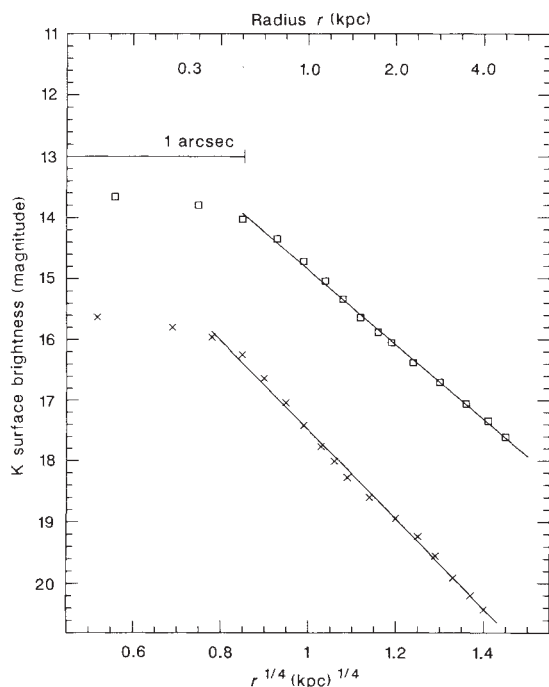


FIG. 1 K surface brightness (mag per arcsec²) plotted against the fourth root of the radius for (□) Arp220 and (×) NGC2623. The scale along the top is radius in kpc, assuming $H_0 = 50 \text{ km s}^{-1} \text{ Mpc}^{-1}$. The plot for NGC2623 has been offset by +2 magnitudes for clarity. Statistical error bars are smaller than the data points. Error bars representing the uncertainty in the subtracted sky level, estimated as described in the text, are plotted for those points where the error bar is larger than the data points. Note that this is a systematic error which will shift all affected points in the same direction.

have recently drawn attention to the two faint crossed tails of Arp220, and the tails of NGC2623 are clearly visible on sky survey plates. Such pairs of tails have been shown in dynamical simulations to be a characteristic of mergers of disk galaxies of approximately equal mass. The K-band images of Arp220 are very different from their optical equivalents as they all have a smooth distribution of light with an apparently single nucleus. This has also been reported for NGC2623, albeit at lower resolution⁹. In Arp220 the location of the nucleus is consistent with that found at K (2.2 μm) by Norris¹⁰.

Several authors^{11–14} have predicted that mergers should develop elliptical-like profiles over a period of $\sim 10^9$ yr, based on n -body simulations of merging disk galaxies. The theoretical foundation for this process is well established but apart from Schweizer's work³, mentioned above, there have been few direct observational tests. To test the hypothesis that these mergers are evolving into elliptical galaxies, we have compared the surface-brightness profiles with those expected for elliptical and spiral galaxies. Figure 1 shows profiles for Arp220 and NGC2623, in which the K-band surface brightness (in magnitudes) is plotted against $r^{1/4}$ where r is the radius. The characteristic de Vaucouleurs law¹⁵ for elliptical galaxies is a straight line on this plot. The lines shown were obtained by a least-squares fit to all points except for the inner two, which are artificially reduced because of seeing and decentring effects. The lines are clearly a good fit to the data over a range of about 4 magnitudes and over linear scales of about 5 kpc in radius, using Hubble's constant $H_0 = 50 \text{ km s}^{-1} \text{ Mpc}^{-1}$. The similarity with elliptical galaxies is strengthened by the half-light scale lengths for the two mergers, which we derived from the best-fit de Vaucouleurs law profiles. The effective radius containing half the K-band

light is 1.6 kpc for NGC2623, and 3.4 kpc for Arp220, consistent with published values for elliptical galaxies¹⁶. Figure 2 shows surface brightness plotted against linear radius for Arp220 and NGC2623 compared to a pure $r^{1/4}$ law and to the profile of the face-on Sc spiral galaxy NGC628 which has a dominant exponential disk¹⁷. In Fig. 2, the profiles of Arp220 and NGC2623 resemble the $r^{1/4}$ law much more closely than they do the spiral galaxy. This is consistent with Fig. 1 in which the faintest points plotted do not deviate systematically from the $r^{1/4}$ law even out to 5 kpc in radius, but the limited area covered by these data makes it impossible to rule out the existence of a more extended disk. We conclude, on the basis of our present data, that Arp220 and NGC2623 have stellar light profiles which bear an intriguingly close resemblance to elliptical galaxies.

K-band images for the remaining four galaxies show light profiles that are not simply those of spiral or elliptical galaxies. Two of these, IC883 and NGC4194 are well fitted by an $r^{1/4}$ law beyond ~ 1 kpc, but the profile is significantly flattened within this radius. NGC6240 and NGC6052 differ from elliptical or spiral galaxies over the entire surface-brightness range and it may be significant that these four galaxies all have nuclei substantially offset from the outer isophotes. These four galaxies are quite unlike Arp220 and NGC2623, which follow an $r^{1/4}$ law over four magnitudes in surface brightness. This suggests that Arp220 and NGC2623 are among the most dynamically evolved of the merger remnants associated with IRAS sources.

Although Arp220 and NGC2623 seem to have developed a mass distribution similar to that of an elliptical galaxy, they have substantially more cold gas and dust than elliptical galaxies. Any scenario for the evolution of mergers into ellipticals must account for the removal of this interstellar material. Both Arp220 and NGC2623 are examples of galaxies in which the merger is thought to have induced exceptionally luminous infrared emission^{6,8} either from an intense starburst or by fueling nuclear activity. Such activity must play an important role in the evolu-

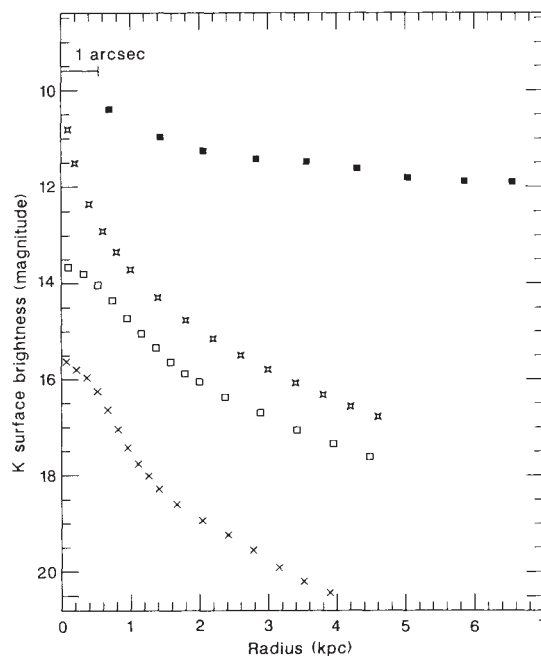


FIG. 2 K surface brightness (mag per arcsec²) plotted against linear radius (kpc) for (□) Arp220 and (×) NGC2623. The NGC2623 data are again offset by +2 magnitudes. Also shown are plots for a pure $r^{1/4}$ law (—) and the Sc spiral galaxy NGC628 (---) taken from the data in ref. 14. The scale is radius in kpc, assuming $H_0 = 50 \text{ km s}^{-1} \text{ Mpc}^{-1}$. Statistical error bars are smaller than the data points.

tion of the remnant. For example, Graham *et al.*¹⁸ have shown that the supernovae which result from starbursts can provide sufficient mechanical energy to leave the galaxy severely gas depleted. Similarly, it has been suggested (refs 19, 20 and R. G. Carlberg, manuscript in preparation) that a quasar-like active nucleus could consume most of the remaining gas on a timescale significantly shorter than a Hubble time. It seems likely therefore that the high IRAS luminosities of Arp220 and NGC2623 are an indication that they will eventually become as free of cold gas as the ellipticals they resemble at K.

It is clear that the relation between degree of relaxation and

other evolutionary processes in mergers is one of the most important questions in the study of present-epoch galaxy evolution. Although we recognize that there are significant problems with making all ellipticals from mergers (see, for example, ref. 21), our results for NGC2623 and Arp220 support the view that merger remnants may evolve into ellipticals. Extending this analysis to a larger sample of mergers and going to fainter isophotes will provide the detailed picture necessary for a more definitive test of the idea that mergers can transform spirals into elliptical galaxies. □

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Evidence for redox cycling of iron in atmospheric water droplets

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ALTHOUGH liquid water is a minor component of the troposphere, the chemical reactions that occur in water droplets (clouds, fog or rain) affect the composition of the atmosphere and the atmospheric input to terrestrial and aquatic ecosystems. Iron (Fe(II) and Fe(III)) is involved in many of these processes, particularly redox and radical chain reactions^{1–6}. Here we report measurements of Fe(II) concentration (up to 200 μM) in fog water (pH 3–7). We find that a large fraction of the total Fe is present as dissolved Fe(II). The concentrations of Fe(II) increase both with exposure to light and with decreasing pH. The concentrations of dissolved iron in fog droplets are much higher than those measured in oxic surface waters and so atmospheric deposition may be a source of dissolved Fe(II) to surface waters, thereby increasing the availability of iron to aquatic biota.

Atmospheric water droplets (cloud, rain or fog droplets) represent a reactive medium in which numerous chemical reactions, especially oxidation (for example, of SO_2 or organic components) by the available oxidants (O_2 , O_3 , H_2O_2 , $\text{OH}^\bullet$) occur. Fog water contains much higher concentrations of dissolved components (such as sulphate, nitrate, chloride, sulphite and ammonium) than rain water^{7,8}. High concentrations of total metals such as Fe, Mn, Cu and Pb⁸ as well as of a variety of organic compounds⁹ have also been measured in fog water.

Here we investigate the redox cycling of iron in fog water. Atmospheric iron may be derived from both natural (transport of soil particles) and anthropogenic (fossil fuel and solid wastes combustion) sources. The iron contributed by these sources is present mainly as Fe(III) oxides. In fog water collected at a sampling site in a suburb of Zürich during a winter and autumn period, dissolved Fe(II) constituted 20–90% of the total Fe

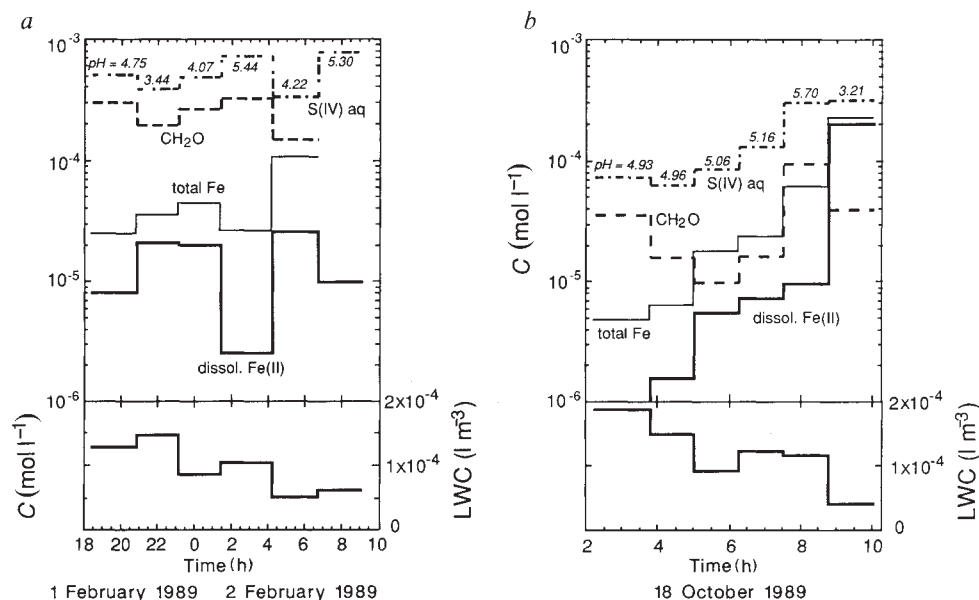
(including particles) and Fe(II) was the predominant oxidation state in solution (Fig. 1). Reductants such as S(IV) or CH_2O were also present in fog water. The effect of pH is shown in Fig. 1a. Decreases in pH coincided with increased concentrations of Fe(II) and vice versa. A great increase in the concentration of dissolved Fe(II) was observed following exposure to light whereas the concentration of total Fe per volume of air did not vary with the liquid-water content (Fig. 1b). In this case, the effect of light is enhanced by a decrease in pH (pH 3.2, $[\text{Fe(II)}] = 200 \mu\text{M}$).

Such high concentrations of Fe(II) in fog water are unexpected, as Fe(II) is thermodynamically unstable in the presence of oxygen and other oxidants. A possible reaction scheme for the Fe(II)/Fe(III) cycle is outlined in Fig. 2. Fe(III) is reduced by sulphite, aldehydes, radicals ($\text{HO}_2^\bullet/\text{O}_2^\bullet$, $\text{RO}_2^\bullet$), Cu(I) and under certain conditions by H_2O_2 (refs 2–6). Other organic compounds may also act as reductants. Efficient photochemical reactions are known, such as the photochemical reduction of oxalato or hydroxy Fe(III) complexes¹. The oxidation reactions of Fe(II) are by O_2 , O_3 and H_2O_2 in the dark, and by H_2O_2 , $\text{HO}_2^\bullet/\text{O}_2^\bullet$ and $\text{OH}^\bullet$ in the light. The pH-dependent solubility of Fe(III) (hydr)oxides is enhanced in the presence of suitable ligands.

The concentrations of the various Fe species depend on the relative rates of the competing processes shown in Fig. 2 and thus on the presence of light, pH and concentrations of reactive species. Reductive dissolution of Fe(III) oxides by sulphite or organic ligands increases the dissolution rates which depend on the crystalline state and the specific surface area^{10–12}. In the presence of light, photochemical reactions lead to increased production of Fe(II). The rate of homogeneous oxidation of Fe(II) by O_2 is strongly pH-dependent and becomes very slow at pH < 6: for example, at 5 °C, half-times for oxidation of Fe(II) are 4.6 years at pH 5, and 126 days at pH 6 (ref. 13). The oxidation by O_3 or H_2O_2 is significant in this pH range although their concentrations in water in equilibrium with the gas phase are low.

In a simple model based on this scheme, we have assumed that a steady-state concentration of Fe(II) is reached in fog water as the result of a balance between reduction and oxidation with excess Fe(III) present in particulate form. This steady-state concentration is pH-dependent through the pH-dependence of

FIG. 1 Concentrations of iron (dissolved Fe(II) and total Fe), formaldehyde, sulphite, pH (numbers above the upper line), and liquid-water content (LWC) in fog-water samples plotted against time for two fog events. Sampling (collection time: 1–2 h) was performed with a screen collector²¹ (18 October 1989: temperature (T) = 5 °C) or with a cyclone collector²² in which the fog was collected as an icy layer (1 February 1989: T = –3 °C). Results from both systems were comparable. These selected data exemplified the role of pH and light (18 October 1989: sunrise at 6:48) in iron reduction. S(IV) may reduce Fe(III) to dissolved Fe(II). Filtered aliquots immediately acidified (to 0.6 M–1.0 M H_2SO_4) for Fe(II) determination, which was carried out within ~1 h of the sampling by the ferrozine spectrophotometric method²³ (detection limit: 0.2 μM); interference with Fe(III) was avoided by complexation with EDTA (ethylenediaminetetracetate)²⁴, and the measurement done within a few minutes. Total Fe (including particulate Fe) and dissolved Fe (by filtration at 0.45 μm and 0.05 μm) determined by flame atomic absorption spectrophotometry (filtered samples acidified with 0.01 M HNO_3). S(IV) measured by ion chromatography did not include hydroxymethanesulphonate (HMSA). Concentrations of formaldehyde, determined by fluorometry²⁵, included a



fraction bound as HMSA, and were always less than the concentrations of S(IV). The pH measured immediately at the temperature of fog water or at 5 °C (calibration by Gran plot). LWC reflects the density of fog water in the atmosphere.

Fe(III) oxide solubility, of many of the rate constants and of the concentrations of reactive species. The following expression for the steady-state concentration is obtained:

$$\log [Fe(II)] = \log (K_s([H^+]^3 + K_1[H^+]^2 + \beta_2[H^+])) + \log (k_{S(IV)}[S(IV)]_{aq}^2 + (k_{HO_2} + k_{O_2}K[H^+]^{-1})[HO_2] + k_{H_2O_2}[H_2O_2] + k_{Cu}[Cu(I)]) - \log (k'_{O_3}[O_3] + k'_{O_2}p_{O_2} + (k'_{HO_2} + k'_{O_2}K[H^+]^{-1})[HO_2] + k'_{H_2O_2}[H_2O_2]) \quad (1)$$

Fe(III) oxide dissolution
term due to reduction of Fe(III)
term due to oxidation of Fe(II)

where K_s is the solubility product of Fe(III) oxides; K_1 , β_2 are the hydrolysis constants of Fe(III); K is the acidity constant of HO_2/O_2^- ; and k_i are the rate constants for reaction of Fe(II) or Fe(III) with species i . The reduction of Fe(III) by CH_2O is assumed to be negligible⁴.

The calculated steady-state concentrations of Fe(II) as a function of pH are shown by the solid lines in Fig. 3 for two cases, with and without light. The computations were made with measured concentrations of S(IV), Cu and O_3 . The concentrations of radicals and H_2O_2 are assumed to be lower during the night than during the day^{14,15}. As we assume that the free Cu(I) is involved in the reduction of Fe(III), the role of Cu(I) becomes negligible. Thus, the steady-state concentration of Fe(II) is lower in the dark than in the light. Despite considerable scatter, concentrations of Fe(II) (closed circles) measured in different fog samples show a pH dependence in agreement with this calculation. Daytime or early-evening samples generally have higher concentrations of Fe(II) than night-time samples. This effect is due to a decrease in the concentrations of radicals and H_2O_2 in the dark. In the absence of light, the reduction of Fe(III) by sulphite, which depends on pH (ref. 6), is important. The reaction rate of Fe(III) with sulphite is still imprecisely known

with respect to the rate of Fe(II) production^{6,16}; the presence of SO_4^{2-} or $S_2O_6^{2-}$, and Fe(II) were, however, shown at very low pH (ref. 17). During daytime, the radicals HO_2/O_2^- and $OH^\bullet$ are the most important. Finally, the high concentrations of Fe(II) measured agree with the reaction scheme assumed, although uncertainties remain with regard to the reaction rates.

As the reduction mechanisms postulated for the reduction of Fe(III) to Fe(II) involve commonly occurring compounds such as S(IV), radicals and H_2O_2 , the same processes may be efficient in producing dissolved Fe(II) in other places. High total concentrations of Fe were observed in fog waters from California⁷ and from Italy¹⁸. Rain water at our sampling station was found

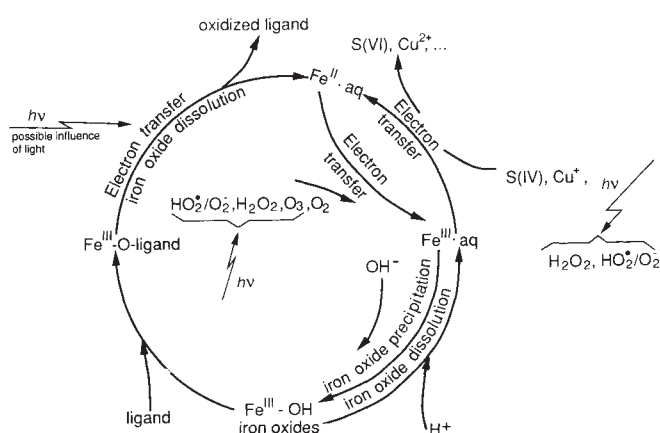


FIG. 2 Reaction scheme for Fe(II/III) in the dark and/or in the presence of light for atmospheric water. Fe is an important electron-transfer catalyst, which affects numerous reactions such as the oxidation of SO_2 , reactions with H_2O_2 and with radicals. Its role in all these reactions will strongly depend on its oxidation state and speciation.

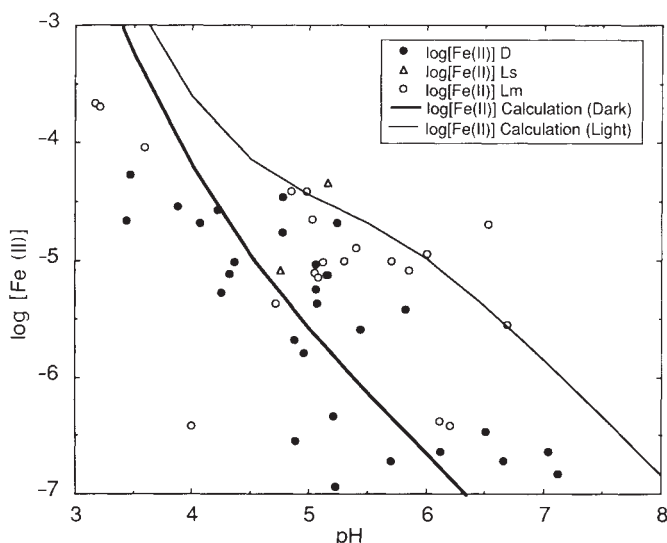


FIG. 3 Comparison of Fe(II) measured in fog samples and Fe(II) calculated for a steady-state assumption, as functions of pH. Ordinate is log scale concentration measured in mol l^{-1} . D (●) represents samples collected in the dark during night-time; Lm (○) samples collected during morning hours with daylight; Ls (△) samples collected in the evening within few hours after sunset. Calculation using equation (1): (—) during night-time; (—) in the presence of light. Assumptions: $T = 5^\circ\text{C}$, $K_s = 1.3 \times 10^6 \text{ l}^2 \text{ mol}^{-2}$; $K_1 = 1.2 \times 10^{-3} \text{ mol l}^{-1}$; $\beta_2 = 6.8 \times 10^{-8} \text{ mol}^2 \text{ l}^{-2}$; $K_{\text{S(IV)}} = 26 \text{ l}^2 \text{ mol}^{-2} \text{ s}^{-1}$; $k_{\text{HO}_2} = 7.8 \times 10^3 \text{ l mol}^{-1} \text{ s}^{-1}$; $k_{\text{O}_2} = 1.0 \times 10^8 \text{ l mol}^{-1} \text{ s}^{-1}$; $K = 2 \times 10^{-5} \text{ mol l}^{-1}$; $k_{\text{H}_2\text{O}_2} = 60 \text{ l mol}^{-1} \text{ s}^{-1}$; $k_{\text{Cu}} = 1 \times 10^6 \text{ l mol}^{-1} \text{ s}^{-1}$; $k'_{\text{O}_2} = 1.7 \times 10^4 \text{ l mol}^{-1} \text{ s}^{-1}$; $k'_{\text{O}_2} = 1.2 \times 10^{-9} + 2.2 \times 10^{-13} [\text{H}^+]^{-1} + 9.9 \times 10^{-20} [\text{H}^+]^{-2} \text{ (atm}^{-1} \text{ s}^{-1})$; $k'_{\text{HO}_2} = 6.2 \times 10^5 \text{ l mol}^{-1} \text{ s}^{-1}$; $k'_{\text{O}_2} = 6.1 \times 10^6 \text{ l mol}^{-1} \text{ s}^{-1}$; $k'_{\text{H}_2\text{O}_2} = 7.6 \text{ l mol}^{-1} \text{ s}^{-1}$ (refs 2–4, 6, 13); $[\text{S(IV)}]_{\text{aq}} = 400 \text{ } \mu\text{M}$; $p_{\text{O}_2} = 0.2 \text{ atm}$; $[\text{O}_3] = 1 \text{ nM}$; $[\text{Cu(II)}] \approx 0 \text{ nM}$. At night, $[\text{HO}_2] \approx 0 \text{ M}$; $[\text{H}_2\text{O}_2] = 1 \text{ } \mu\text{M}$; during daytime, $[\text{HO}_2] = 5 \times 10^{-11} \text{ M}$; $[\text{H}_2\text{O}_2] = 30 \text{ } \mu\text{M}$ (concentrations estimated^{14,15}); an excess of Fe(III) in particulate form was assumed. As concentrations of total concentration of Fe in samples did not exceed $300 \text{ } \mu\text{M}$, $\log [\text{Fe(II)}]$ cannot be larger than -3.5 .

to contain $0.5\text{--}2 \text{ } \mu\text{M}$ total Fe and detectable Fe(II). Substantial concentrations of Fe were measured in aerosols, even at remote locations in the Pacific¹⁹.

Thus, dissolved Fe(II) in atmospheric water could have a significant impact on iron cycling in natural waters. It has been recently argued that Fe is a limiting nutrient in the oceans²⁰ and the supply of Fe in soluble form from atmospheric deposition could enhance phytoplankton productivity. □

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A superplastic covalent crystal composite

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SUPERPLASTICITY is defined phenomenologically as the ability of a material to exhibit exceptionally large elongations during tensile deformation¹. It is a property of some polycrystalline solids, and is well established for metals and alloys². Superplasticity has also been observed in some ionic crystals, such as Y_2O_3 -stabilized tetragonal ZrO_2 polycrystals^{3,4}, but has not been found previously for covalent crystals. Here we report superplastic elongation (by more than 150%) of a covalent crystal composite, $\text{Si}_3\text{N}_4/\text{SiC}$. The superplasticity is probably related to the presence of an intergranular liquid phase. Combined with its hardness, this property suggests several useful applications for the novel material: for example, to form engine components—superplasticity will make it readily mouldable at high temperatures.

The deformation mechanism of micrograin superplasticity is grain-boundary sliding. When hard grains slide over each other, the formation of cracks and cavities at grain boundaries is inevitable. Some kind of accommodation process at three-grain junctions is required for polycrystalline materials to be elongated continuously without fracture. For superplastic metals, accommodation mechanisms involving either diffusion⁵ or dislocation motion⁶ have been considered.

The mechanism of grain-boundary sliding depends on the

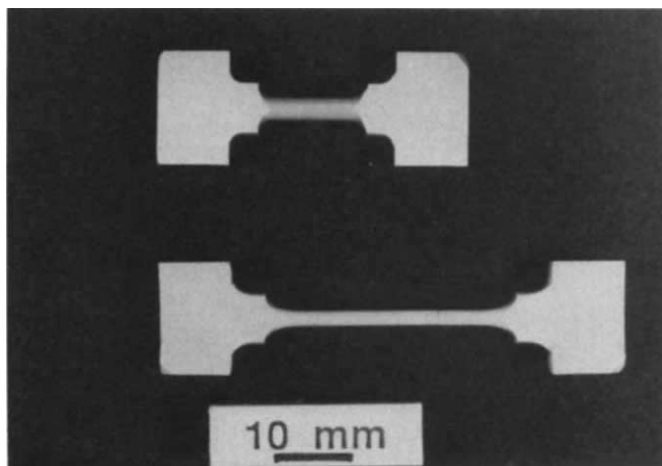


FIG. 1 Undeformed and superplastically elongated $\text{Si}_3\text{N}_4/\text{SiC}$ composite specimens ($1,600^\circ\text{C}$; initial strain rate, $4 \times 10^{-5} \text{ s}^{-1}$).

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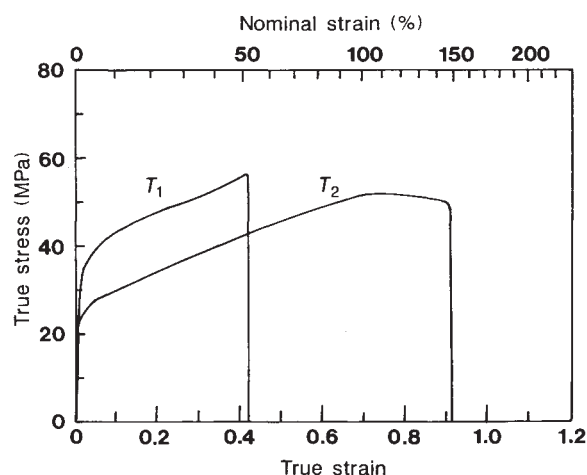


FIG. 2 True-stress/true strain curves at 1,600 °C. (Initial strain rates $T_1 = 8 \times 10^{-5} \text{ s}^{-1}$, $T_2 = 4 \times 10^{-5} \text{ s}^{-1}$.)

structure of the grain boundary. A liquid phase present at grain boundaries will enhance sliding⁷. Wang and Raj⁸ predicted that superplasticity might occur in liquid-phase-sintered Si_3N_4 by diffusional creep enhanced by solution and precipitation of crystals in a Si-O-N liquid phase at the grain boundaries. But superplastic tensile elongation has not been achieved in this material, even though high ductilities⁹ in compression have been observed, because cavitation at grain boundaries under tension and subsequent fracture occurs readily in the presence of an intergranular liquid phase. The maximum elongation reported so far in creep deformation of Si_3N_4 is <8%. (ref. 10).

We have studied the superplasticity of a $\text{Si}_3\text{N}_4/\text{SiC}$ composite. The starting powder was prepared by vapour-phase reaction^{11,12} of $[\text{Si}(\text{CH}_3)_3]_2\text{NH}/\text{NH}_3/\text{N}_2$ at 1,000 °C. The powder was heat-treated in N_2 at 1,350 °C for 4 h. The amorphous Si-C-N powder (Si, 58 wt%; C, 7 wt%; N, 33.9 wt%; O, <1 wt%; Fe, Al, Ca, <50 p.p.m.) was mixed with 6 wt% Y_2O_3 and 2 wt% Al_2O_3 as sintering aids. The mixture was hot-pressed at 1,650 °C for 0.7 h at 34 MPa in N_2 . The density of the composite was 3.2 g cm^{-3} . Fracture toughness and strength at room temperature were $5.5 \text{ MN m}^{-3/2}$ and 672 MPa respectively.

X-ray analysis revealed that the composite was composed mainly of $\beta\text{-Si}_3\text{N}_4$, $\alpha\text{-Si}_3\text{N}_4$ and $\beta\text{-SiC}$ grains (~20 wt%). An X-ray diffraction peak characteristic of $\text{Si}_3\text{N}_4 \cdot \text{Y}_2\text{O}_3$ was also detected. The ratio of β -phase to total Si_3N_4 was 67%. Scanning electron microscopy showed the presence of spherical grains (diameter <200 nm) and elongated grains (<500 nm). The added Y_2O_3 and Al_2O_3 reacted with Si_3N_4 to form a liquid during hot-pressing. Part of the liquid phase crystallized as $\text{Si}_3\text{N}_4 \cdot \text{Y}_2\text{O}_3$ and the remnant remained as a glassy phase after cooling. The composition of the glassy phase was not determined.

Tension tests at constant cross-head speeds were conducted in N_2 (1 atm) at 1,600 °C. It was supposed that at this temperature the glassy phase formed a liquid¹³ composed of Si, O, N, Al and Y at two- and three-grain junctions. Uniform superplastic elongation (>150%) was achieved (Fig. 1). We define nominal strain (ϵ) and true strain (ϵ_t) as

$$\epsilon = (L - L_0)/L_0 \quad (1)$$

$$\epsilon_t = \ln(L/L_0) \quad (2)$$

where L and L_0 are the elongated and the original gauge length, respectively. The true-stress/true-strain curves at different initial strain rates are shown in Fig. 2. Larger elongation leading to failure was possible at lower strain rates. The constitutive equation for steady creep can generally be expressed as

$$\dot{\epsilon} = A\sigma^n/d^p \quad (3)$$

where $\dot{\epsilon}$ is the strain rate, σ is the stress, d is the grain size, p and n are grain-size and stress exponents, and A is a coefficient of proportionality. A steady state could not be achieved because strain hardening (flow stress increasing with deformation) was observed (Fig. 2). From the relationship between flow stress at a true strain of 0.1 and strain rate, we obtain $n \approx 2$. Models of diffusional creep enhanced by a liquid phase predict $n = 1$, and thus the superplasticity observed cannot be explained by such models^{7,8}.

A scanning electron micrograph of a fractured surface of the deformed specimen is shown in Fig. 3. We see that the elongated grains have undergone grain growth. X-ray diffraction indicated that a phase transformation from $\alpha\text{-Si}_3\text{N}_4$ to $\beta\text{-Si}_3\text{N}_4$ had occurred, and X-ray peaks from $\text{Si}_3\text{N}_4 \cdot \text{Y}_2\text{O}_3$ and other crystalline phases in the Y-Si-Al-O-N system had increased. Weight loss was also observed during testing. The microstructural changes during deformation were as follows: (1) grain growth of $\beta\text{-Si}_3\text{N}_4$; (2) crystallization¹³ of the intergranular liquid phase; (3) volatilization^{13,14} of the liquid phase. We suggest that the apparent strain hardening can be attributed to these microstructural changes.

The superplasticity exhibited by these covalent polycrystals was characterized by non-newtonian flow. The liquid-phase-enhanced creep model must be modified to accommodate this fact. A stress exponent $n = 2$ has been observed in superplastic alloys¹ that do not contain an amorphous phase, and in zirconia polycrystals (Y-TZP)^{3,4} with a very thin amorphous film at

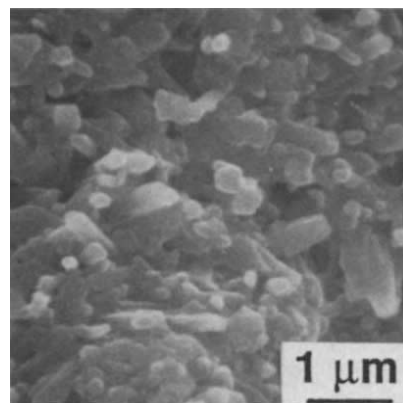


FIG. 3 Scanning electron micrograph of fractured surface of a composite specimen (tested at 1,600 °C for 30 min).

two-grain junctions, as well as in our $\text{Si}_3\text{N}_4/\text{SiC}$ composites. Thus non-newtonian flow may be a common feature of superplasticity in fine-grained polycrystalline materials.

Si_3N_4 and SiC are promising structural materials for mechanical applications, especially in forming wear-resistant components such as engine parts, because of their excellent mechanical properties such as strength and hardness. Plastic working of covalent crystals by dislocation glide is difficult because of their high Peierls force; but we have shown that superplasticity is possible in covalent polycrystals. All the applications of superplasticity (such as superplastic forming) can therefore be applied to covalent polycrystals as well as metals. $\square$

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Structural modelling of glasses using reverse Monte Carlo simulation

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ONE of the main difficulties in the study of glasses and other disordered materials is the production of structural models that agree quantitatively with diffraction data. In normal Monte Carlo simulation, an initial structure is allowed to rearrange in such a way that its energy is minimized. Reverse Monte Carlo simulation¹ is a newly developed technique in which a structural model is adjusted so as to minimize instead the difference between the calculated diffraction pattern and that measured experimentally, so that good agreement is inevitable. No interatomic potential is required. Here we illustrate the potential of this method by fitting the structure of vitreous silica simultaneously to X-ray and neutron diffraction data. The result, a (mostly) continuous random network of corner-sharing SiO_4 tetrahedra, is consistent with other models but, unlike them, is derived solely from the data.

The structure of vitreous silica is generally proposed to be a continuous random network of corner-sharing SiO_4 tetrahedra. Numerous methods have been used to model the structure of this and other glasses. The most important are: (1) hand-built ('ball and stick') models², whose coordinates may be fed into a computer and energy-minimized subject to a chosen interatomic potential³; (2) computer models generated by topological transformation⁴ and subsequent energy minimization of an initial structure⁵; and (3) molecular dynamics (MD) or Monte Carlo (MC) simulations⁶. The best of these agree reasonably well at short range (interatomic distances $r < 10 \text{ \AA}$) with radial distribution functions derived from X-ray⁷ or neutron⁸ diffraction measurements, and a few show some agreement at intermediate range ($10 < r < 20 \text{ \AA}$) or with experimental structure

factors. However, no model has yet been constructed that provides quantitative agreement with the experimental data as measured, that is, in the form of structure factors for both neutron and X-ray diffraction. This severely limits progress in understanding the structure of vitreous silica and of glasses in general⁹.

The methods used so far for model building all have specific disadvantages. Hand-built models are subjective, difficult to construct and modify, and have problems associated with surface atoms because of the finite size of the model. Energy-minimization computer models and MD and MC simulations generally use periodic boundary conditions, thus eliminating the complications of surfaces, but they are dependent on the choice of interatomic potential; this often involves some form of angle dependence that determines the angular correlations. Topological models depend strongly on the choice of initial structure. The reverse Monte Carlo (RMC) method¹ is based on the Metropolis Monte Carlo algorithm, but instead of minimizing the energy, one minimizes

$$\chi^2 = \sum_k \sum_i (F_k^C(Q_i) - F_k^E(Q_i))^2 / 2\sigma_k^2(Q_i)$$

that is, the difference between the experimental structure factor(s), $F_k^E(Q_i)$, and those calculated from the simulation configuration, $F_k^C(Q_i)$. Here $\sigma_k(Q_i)$ is an estimate of the error in $F_k^E(Q_i)$. The structure is modified in this way until it agrees with the experimental data within some satisfactory measure of the error. (The assessment of 'satisfactory' agreement relies on a knowledge of possible sources of error in the experimental method, in the particular data used and in its treatment.) This method is simple and has none of the disadvantages noted above for other methods. It does have the obvious disadvantage in comparison with MD or MC simulation that it provides only static structural information: no information about the thermodynamics or dynamics of the system can be gained. If good interatomic potentials are available, MD simulation should be used instead of RMC. There are numerous disordered materials, however, for which suitable potentials have not been developed; the structural model generated by RMC could possibly be used in these cases to provide an indication of how to modify the existing potentials.

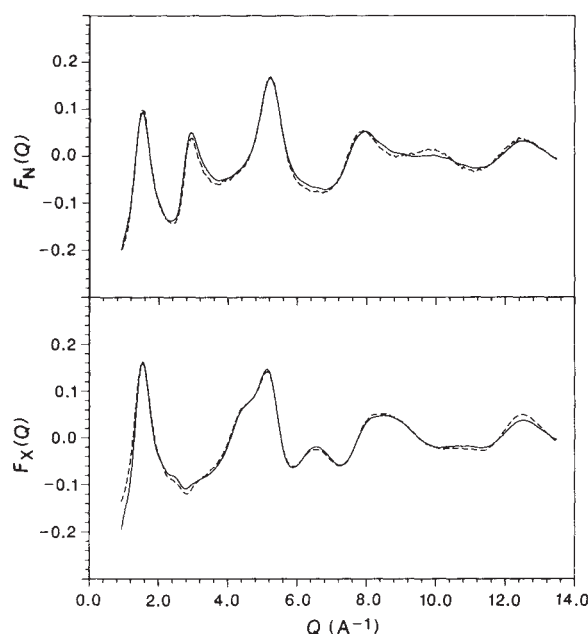


FIG. 1 Structure factors $F(Q)$ for vitreous silica. *a*, Neutron diffraction, and *b*, X-ray diffraction. The dashed line is experimental, and the solid line is the RMC fit.

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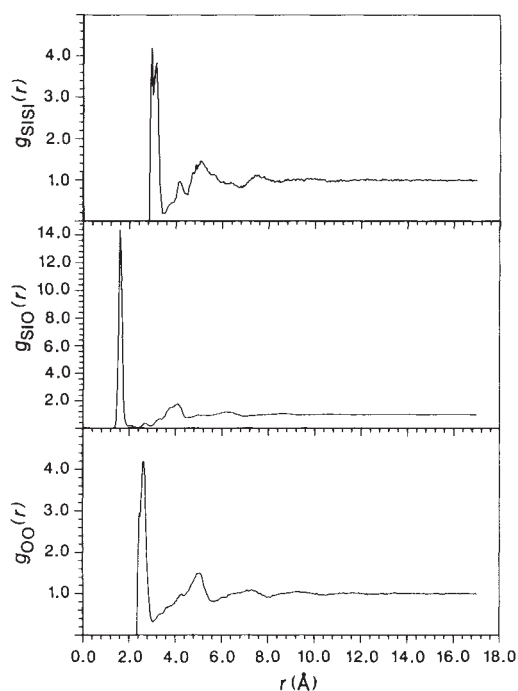


FIG. 2 Partial radial distribution functions $g(r)$ for vitreous silica.

For modelling the structure of vitreous silica we have used a configuration of 2,596 atoms in a cube of side $L = 34.017$ Å with periodic boundary conditions, starting from a random arrangement of hard spheres. Note that it is important, particularly when modelling materials with significant intermediate-range order such as network glasses, to compare experimental and

calculated structure factors rather than radial distribution functions, so a large number of atoms must be used. RMC and MC simulations are significantly faster than MD in this respect. The constraints on the model are the experimental density and the closest distances that two atoms are allowed to approach. These have been set at $r_{\text{SiO}} = 1.35$ Å, $r_{\text{OO}} = 2.4$ Å and $r_{\text{SiSi}} = 2.9$ Å. r_{SiO} and r_{OO} may be found directly, and a good estimate of r_{SiSi} made, from the experimental radial distribution functions. r_{SiSi} may also be calculated from the requirement that no SiO_4 tetrahedra are edge-sharing, as indicated by NMR results¹⁰. These parameters may therefore be considered as experimental and are not modified to bias the model towards a structure with particular features.

The experimental neutron and X-ray structure factors, measured on the same sample under the same conditions (J. B. van Tricht, personal communication) are compared with those calculated by RMC (averaged over 10 configurations) in Fig. 1. The differences are significantly smaller than those from any previous model and are comparable to those between different experiments⁸. The agreement, however, is not and indeed should not be perfect, because the experimental data will contain errors that, given the constraints on density and closest approach noted above, cannot correspond to a possible physical structure. The partial radial distribution functions, $g_{\alpha\beta}(r)$, derived from the RMC structure are shown in Fig. 2. The coordination number, n_{SiO} , obtained from integration of the first peak in $g_{\text{SiO}}(r)$, is assumed by most models to be 4. Simulations do not usually require $n_{\text{SiO}} = 4$, but the starting configuration will normally have such a coordination number. The best neutron diffraction data⁸ gives $n = 3.86 \pm 0.15$; we find a lower (although consistent within the errors) value of 3.7. Some reduction in coordination is due to the limited resolution of the neutron scattering data (which also leads to a slight split in the first peak in $g_{\text{SiSi}}(r)$), but other factors such as incorrect normalization of the experimental data and the use of the free-atom X-ray form factors in the calculated X-ray structure factor may contribute. The

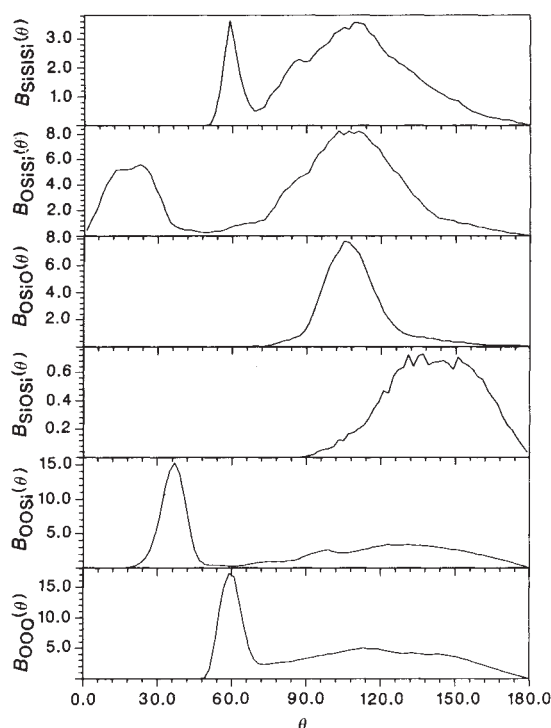


FIG. 3 Bond-angle distributions $B(\theta)$ for vitreous silica. Maximum bond lengths are defined by the first minima in the appropriate partial radial distribution functions.

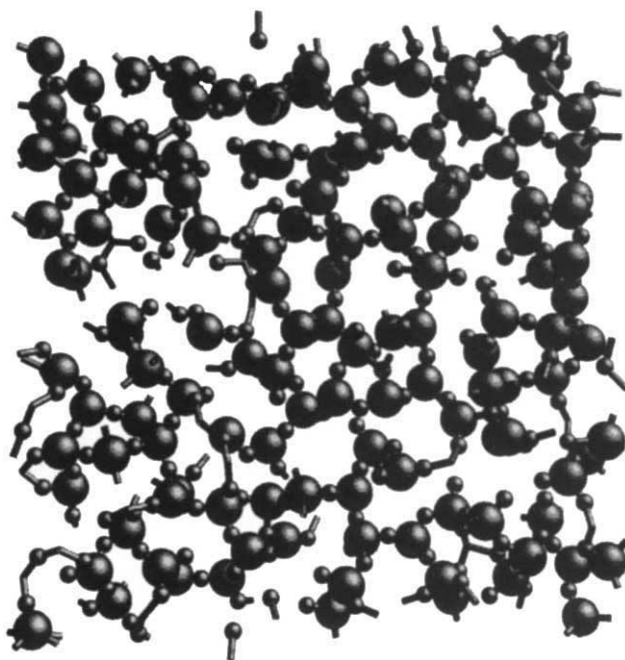


FIG. 4 Projection of atomic positions in a 7-Å-thick section from a representative configuration. Larger atoms are Si, smaller atoms are O. Si-O bonds from all atoms within the section are drawn, so some bonds terminate on atoms outside of the section, which are not drawn.

absolute normalization may be improved by measuring the diffraction pattern over a larger range of Q . Limits on resolution could be accommodated in the RMC procedure by convoluting the calculated structure factors with appropriate functions before comparison with experiment. Coordination-number constraints (from NMR data¹⁰, for example) and EXAFS data (S. J. Gurman, personal communication) could also be included. Given the required increase in computer time, we did not consider these refinements to be worthwhile in this illustrative study. Nevertheless, RMC is able to simultaneously and quantitatively use different experimental information, as demonstrated by the simultaneous modelling of neutron and X-ray structure factors, and this should prove particularly valuable when studying complex systems of technological interest.

Bond-angle distributions $B_{\alpha\beta\gamma}(\theta)$ are shown in Fig. 3. The average O-Si-O bond angle is 109.6° , which is extremely close to the ideal intra-tetrahedral angle of 109.5° . We emphasize that nothing in our model determines the tetrahedral coordination except the experimental structure factors; in most models other than simulations it is pre-determined. The average Si-O-Si bond angle (inter-tetrahedral) of 141° is in close agreement with the value of 144° reported by Mozzi and Warren⁷ from X-ray diffraction, although the distribution is broader. Both B_{OSiSi} and B_{SiSiSi} have peaks close to 109.5° . In addition, B_{SiSiSi} has a sharp peak at 60° which is due to three-membered rings. Many other models do not allow such small rings, although there is nothing in the experimental data to forbid their occurrence and they do occur

in other simulations¹¹. A projection of the atomic positions in a section from the configuration shown in Fig. 4 also contains 4-, 5-, 6-, 7- and 8-membered rings. The general form of the structure is similar to a continuous random network, although some discontinuities are allowed in this model. We have not carried out any further detailed analysis of the structure, such as ring statistics, because we acknowledge that our present model contains deficiencies such as the low value of n_{SiO} .

Thus Reverse Monte Carlo simulation is a powerful method for generating models of the structure of glasses that agree quantitatively with the experimental diffraction data. Constraints or additional information from other experimental measurements may be included easily. We believe that the method will be valuable in studies of disordered materials. $\square$

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A mechanism for particle size segregation in three dimensions

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THE segregation of particles with different properties is important in many areas of science and technology, including materials science, engineering, agriculture and geophysics. In many segregation processes the most important property of the particles is their size. Processes such as pouring, shaking, vibration, shear and fluidization lead to size segregation; in some cases it is found to occur even in processes developed for particle mixing¹. Because of its practical importance, particle segregation has been studied extensively during the past few decades², and several two-dimensional models have been developed recently³⁻⁶. Here we describe results obtained from a simple three-dimensional simulation in which spheres are added, one at a time, to a growing heap on an infinite planar surface or within a cylinder of finite radius. In this model, size segregation is a consequence of the ability of large spheres to roll over a random packing of smaller spheres. Our results show that the degree of segregation is sensitive to both the size ratio and the number ratio for the case of a binary mixture of spheres.

The model used in this work is based on earlier models for the deposition of spherical particles of unequal size onto a flat surface⁷⁻⁹. In this model, particles of radius R_1 and R_2 ($R_2 > R_1$) are dropped randomly onto a growing deposit. After they contact the deposit, they follow the path of steepest descent until either they reach a local minimum on the surface of the deposit or they contact the substrate. At this stage the particles cease

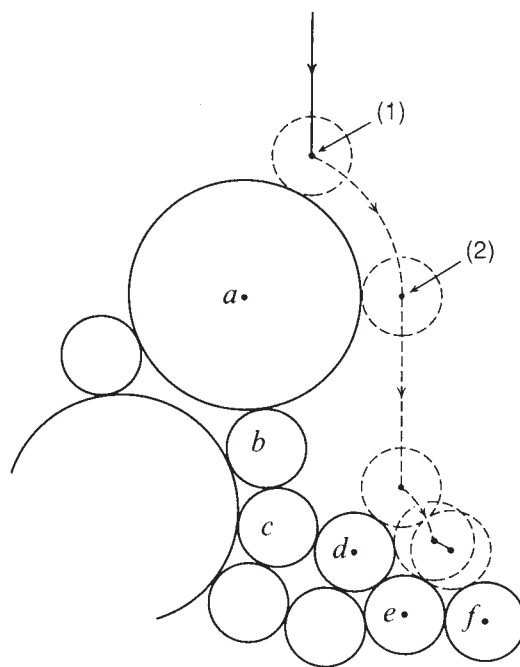


FIG. 1 A two-dimensional representation of the three-dimensional model used in this work. A typical trajectory for a deposited particle is shown. The deposited particle first contacts the deposit at position (1) and then follows a path of steepest descent. At position (2), where the centre of the deposited particle and the centre of the contacted particle in the deposit have the same height, the particle falls freely until the moving particle contacts particle d in the deposit. Finally, the moving particle comes to rest at a local minimum in contact with particles e and f . In the three-dimensional model the particle has reached a local minimum when it contacts three particles in the deposit and the projection of its centre onto the substrate lies within the triangle whose corners are the projection onto the substrate of the centres of the three deposited particles.

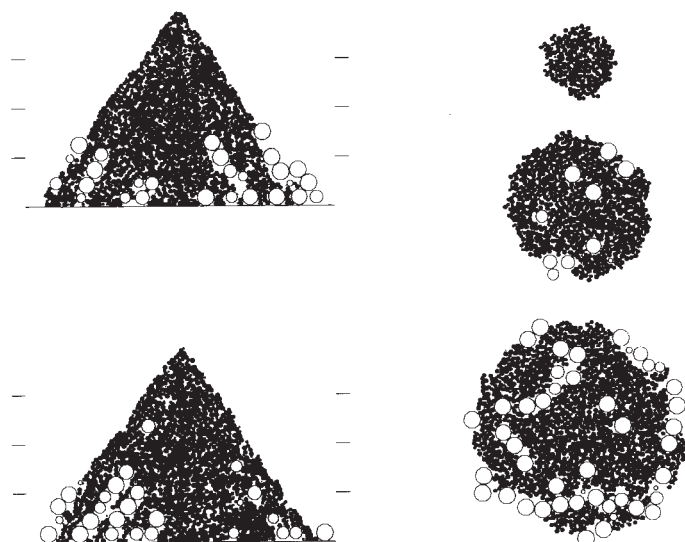


FIG. 2 A heap of 40,206 particles generated using a size ratio (r) of 4 and a number ratio (x) of 0.01. On the left-hand side are two perpendicular vertical cross-sections through the centre of the heap, and on the right-hand side are three horizontal cross-sections at the heights indicated on the left-hand side.

moving and become part of the growing deposit. Figure 1 shows a two-dimensional representation of this model. This three-dimensional model has been used to simulate segregation resulting from a different mechanism, namely the percolation of small particles through the random packing of larger particles^{10,11}. For binary mixtures, this mechanism becomes important only when the ratio $r = R_2/R_1$ becomes comparable to or larger than the apollonian ratio ($R_2/R_1 = (3 + 2\sqrt{3})$) and the fraction of large particles is sufficiently large. In the present model, each of the particles is 'dropped' onto the growing deposit along a vertical trajectory always located at the same lateral position. The deposited particles are selected at random to be large or small with probabilities x and $1 - x$ respectively. The deposited particles either are allowed to form an unrestricted heap on the substrate or are deposited along the axis of a cylinder. In the latter case, the trajectories of the deposited particles end when the particle reaches a local minimum on the surface or contacts the cylinder or the substrate at the base. As the trajectories of the deposited particles are deterministic, the efficiency of the simulations can be improved substantially by making use of information from trajectories followed by particles of the same size that were deposited at an earlier stage. This becomes increasingly important as the lateral extent of the deposits grows and the length of the trajectories becomes longer.

Figure 2 shows a heap of 40,206 particles generated using the model described above with a radius ratio (r) of 4 and a fraction (x) of large spheres of 0.01. This figure shows quite clearly that the large particles are concentrated onto the lower and outer parts of the heap whereas the small particles are concentrated onto the upper part of the heap and into its core.

Figure 3a shows the results of a simulation of the deposition of a binary mixture of particles into the centre of a cylindrical container. In this simulation the radius ratio (r) is 2 and number ratio (x) is 0.1. The large particles are concentrated towards the walls of the container and the small particles are concentrated towards the middle. A more dramatic segregation effect is shown in Fig. 3b. Here the radius ratio (r) has been increased to 4 and the number ratio x has been decreased to 0.01. Another interesting feature that can be seen in this figure is the presence of quite large voids beneath the large spheres (particularly those located near the wall of the cylinder).

To obtain a more quantitative measurement of the particle size segregation effect, we have measured a variety of properties associated with the deposits. These include the vertical and radial number profiles ($n(\rho)/\rho$ and $n(h)$, where ρ is the radial distance from the point of deposition and h is the height above the substrate) for both the large and small spheres. Figure

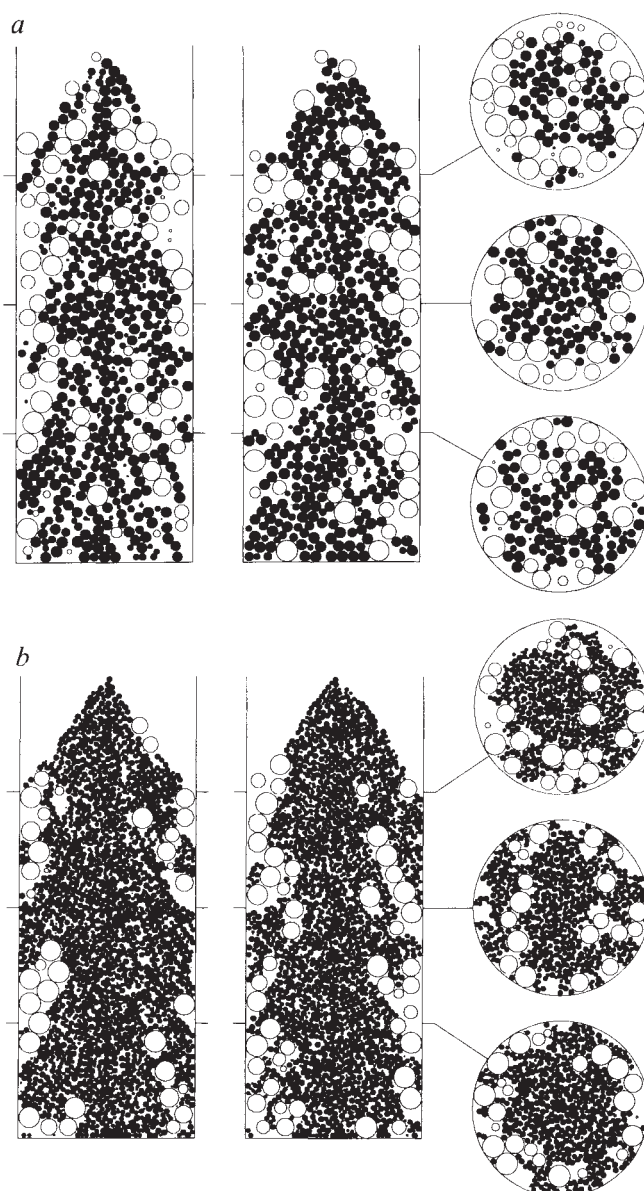


FIG. 3 a, A simulation of the deposition of 4,818 particles into a container of radius $8R_2$. In this simulation, $r=2$ and $x=0.1$. The left-hand side of the figure shows perpendicular cross-sections through the axis of the cylinder and the right-hand side shows cross-sections parallel to the substrate at three different heights. b, As in a, but with $r=4$ and $x=0.01$, and with a total of 40,206 particles.

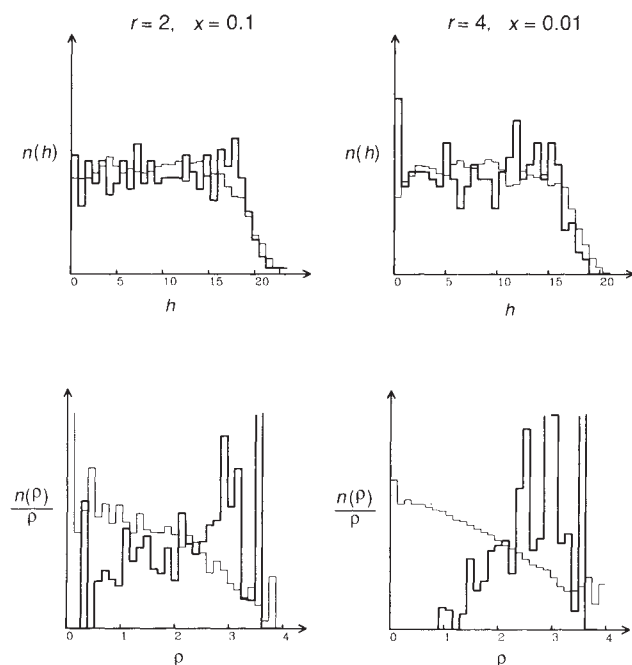


FIG. 4 Particle density profiles obtained from the simulations shown in the respective Fig. 3a and b. The upper plots are vertical profiles ($n(h)$, where $n(h)\delta h$ is the number of particles with centres at heights in the range $h \rightarrow h + \delta h$). The lower plots are radial profiles ($n(\rho)/\rho$, where $n(\rho)\delta\rho$ is the number of particles whose centres are located at distances in the range ρ to $\rho + \delta\rho$ from the axis of the cylinder). The thick and thin histograms correspond to large and small spherical particles, respectively. These profiles have been normalized to equal areas to facilitate comparison.

4 shows the profiles $n(h)$ and $n(\rho)/\rho$ obtained from the simulations illustrated in Fig. 3. These and other simulation results indicate that the degree of segregation increases as r increases and as x decreases. A more quantitative analysis is in progress.

The size segregation mechanism illustrated here is important in processes in which particles are poured into a container or onto a surface. There is a variety of other segregation mechanisms, including percolation^{8,10,11} and segregation brought about by the filling of voids beneath large particles by small particles during shaking and vibration³⁻⁵. Size segregation can also be brought about by shear^{12,13}, freeze-thaw cycling¹⁴⁻¹⁶, saltation¹⁵, fluidization¹⁷ and other processes. Because of these many mechanisms, size segregation is a common phenomenon. Many of these mechanisms can be studied using simple computer models, and we expect that this approach will be employed more extensively in the future using high-speed digital computers. □

Segmentation of the Mid-Atlantic Ridge between 24° N and 30°40' N

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THE Mid-Atlantic Ridge (MAR) extends for over 12,000 km from Iceland to the Bouvet Triple Junction in the south Atlantic. Most of our knowledge of the geology of this slow-spreading centre comes either from widely spaced low-resolution echo-sounding lines or from detailed studies of small areas using deep-towed instruments or submarines. High-resolution multibeam bathymetry^{1,2} has been insufficient to define the variability of accretionary processes along this plate boundary. Here we present the results of a recent investigation of a ~800-km-long section of the MAR carried out using the multibeam echo-sounder SeaBeam³. Analysis of these data reveals the scale and nature of the segmentation of the MAR between the Kane Fracture Zone (24° N) and latitude 30°40' N.

High-resolution mapping of the mid-ocean ridges indicates that spreading centres consist of distinct accretionary segments separated by axial discontinuities⁴. Spreading-centre discontinuities can be classified as transform or non-transform offsets, which differ both in terms of their morphological characteristics and their modes of evolution. The segmentation of mid-ocean ridges⁵⁻⁷ is a global and fundamental phenomenon which reflects the three-dimensional nature of accretionary processes at divergent plate boundaries.

Except for the Atlantis Transform, there are no transform offsets along the ~800-km-long portion of the MAR that we have studied (24°00' N to 30°40' N). Instead, the spreading centre is offset by a series of non-transform discontinuities^{8,9} (Fig. 1). This is contrary to the common assumption¹⁰ that the MAR is periodically offset by transform faults with an average spacing of ~55 km. The non-transform discontinuities observed here are distinct from the ridge-axis discontinuities encountered along the East Pacific Rise (EPR)¹¹, but are similar to the ones present along the MAR in the South Atlantic¹². We have identified 16 spreading segments within our survey area, with lengths that vary from 20 to 85 km. The average spacing of the segments is 53 km. The range of offsets between adjacent segments is from ~0 to 30 km.

The axial depth profile (Fig. 2) of the MAR within our survey area exhibits variations of intermediate wavelength (10–100 km), similar to those observed along the EPR¹³. All of the spreading-centre discontinuities that we observe are located near local depth maxima. Our data suggest a model for the segmentation of the Mid-Atlantic Ridge, and associated length scales, that is conceptually similar to models proposed for the EPR^{11,14}; these two models differ mainly in the morphology of the discontinuities that separate the spreading segments rather than in the processes that cause the segmentation.

We observe rift-valley morphologies ranging from narrow, deep, hourglass-shaped valleys to wide valleys bounded by low-relief rift mountains (Fig. 1). Within each segment, the rift valley is narrowest where the spreading centre is shallowest, and it widens by as much as 10–15 km and deepens by 400–1,200 m near the discontinuities that bound the segments (Fig. 2). The rift valley is often asymmetric: one side may be bounded by steep slopes, which may expose relatively undisturbed sections of oceanic crust¹⁵, whereas the other side may be bounded by gentle slopes.

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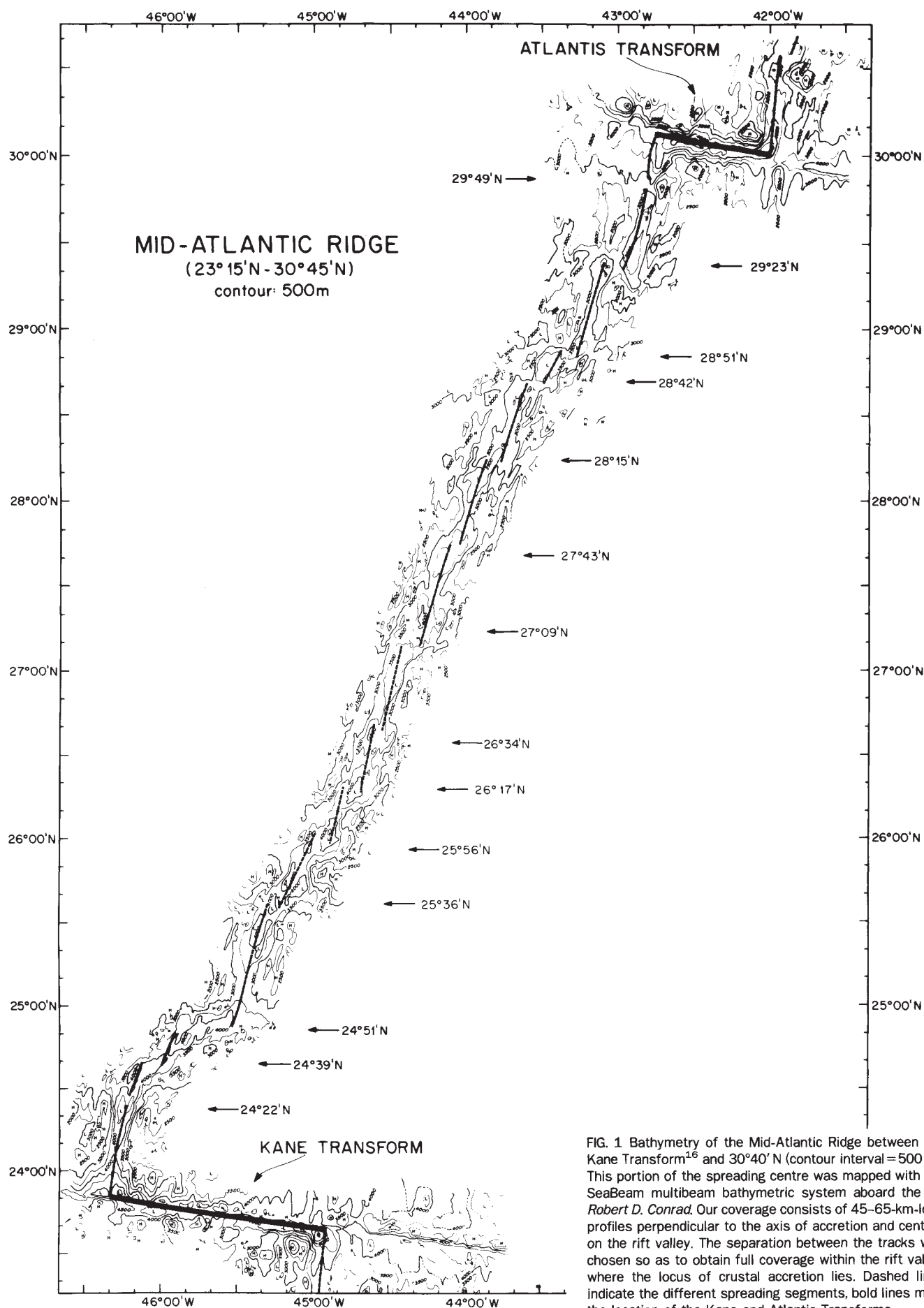


FIG. 1 Bathymetry of the Mid-Atlantic Ridge between the Kane Transform¹⁶ and 30°40' N (contour interval = 500 m). This portion of the spreading centre was mapped with the SeaBeam multibeam bathymetric system aboard the RV *Robert D. Conrad*. Our coverage consists of 45–65-km-long profiles perpendicular to the axis of accretion and centred on the rift valley. The separation between the tracks was chosen so as to obtain full coverage within the rift valley, where the locus of crustal accretion lies. Dashed lines indicate the different spreading segments, bold lines mark the location of the Kane and Atlantis Transforms.

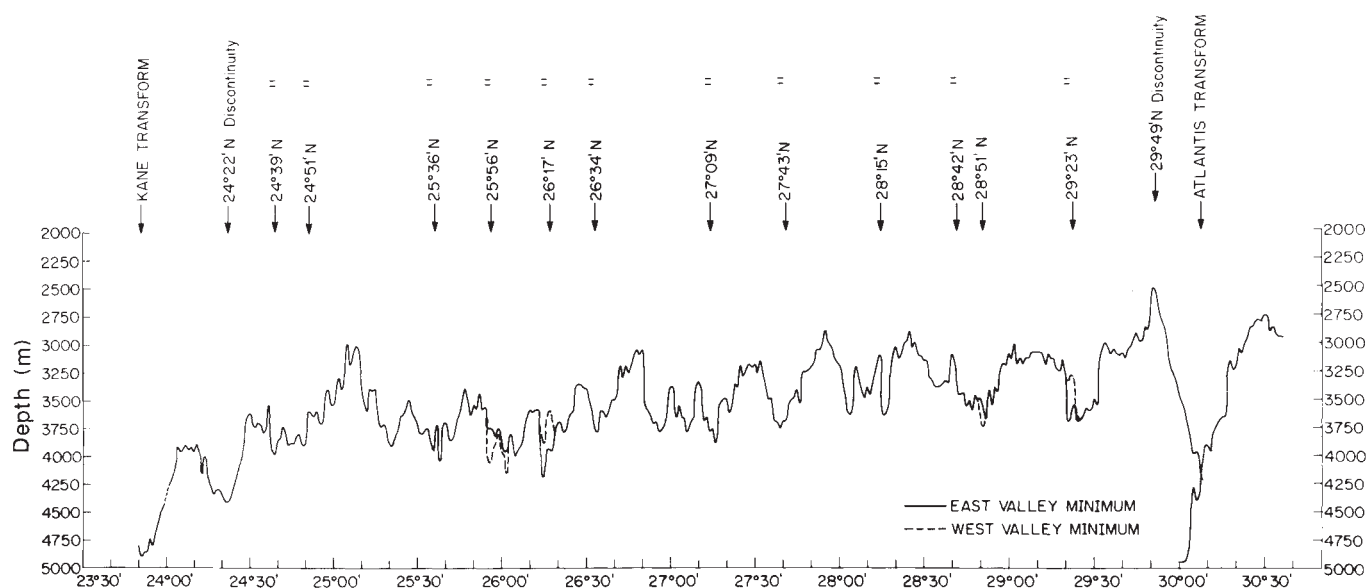


FIG. 2 Along-axis depth variations of the Mid-Atlantic Ridge between the Kane Transform and 30°40' N (minimum depth within the inner floor of the rift valley). The latitude of the spreading-centre discontinuities is indicated. The discontinuities are located near along-axis depth maxima, and bound

the intermediate-wavelength undulations of the spreading centre. The amplitude of depth variations within a spreading segment is approximately 600 m for segments bounded by non-transform discontinuities and 800–1,200 m for those bounded by the Kane or the Atlantis Transform.

The neovolcanic zone typically corresponds to the shallowest portion of the rift valley and is often associated with a ridge flanked by marginal lows. This ridge is generally not centred within the inner floor and its trend may differ slightly from that of the rift valley. The neovolcanic zone is usually more continuous and associated with more numerous volcanic edifices where the spreading centre is shallowest. It loses definition and becomes highly discontinuous toward the distal ends of each spreading segment.

The rift mountains, which rise 400–3,000 m above the valley floor, consist of narrow, axis-parallel ridges that are continuous along strike for up to 60 km (Fig. 1). It has been suggested that these features are constructional volcanic ridges that formed in the rift valley and have been transported into the rift mountains^{1,16}. Our data do not directly support this interpretation: we do not observe the presence within the rift valley of a ridge that is morphologically similar to the ones present in the rift mountains along the 800-km-long portion of the plate boundary that we have mapped. The present magmatic budget of the spreading centre may, however, be lower than in the past, precluding the present-day formation of large neovolcanic ridges on axis.

We suggest that the inter-segment, morphotectonic variability that we observe reflects differences in the volume and temporal continuity of magmatic upwelling at the intermediate-wavelength scale¹⁷. Segments that are experiencing a phase of voluminous and sustained magma upwelling and melt productivity will exhibit a narrow, hourglass-shaped rift valley with a well defined neovolcanic zone. In contrast, segments with a weak magmatic budget will exhibit a wide, U-shaped rift valley with a poorly defined neovolcanic zone. The intra-segment, morphotectonic variations may represent differences in the relative importance of volcanism and tectonism along strike away from localized zone of magma upwelling within each segment. The contribution of volcanism to the morphology will be more important near the shallowest portion of the rift valley within each segment, beneath which we postulate that the upwelling of magma is enhanced, than near the distal ends of the segment. Conversely, the contribution of tectonic extension to the morphology will become more important toward the spreading-

centre discontinuities. The overall variations in the morphotectonic characteristics of the MAR are the result of the superposition of the inter- and intra-segment variabilities.

Within our survey area, we can distinguish several types of spreading-centre discontinuities based on their morphology as well as their spatial and temporal stability. The non-transform discontinuities can be decomposed into second- and third-order discontinuities¹⁴ (Fig. 3), at which the horizontal shear between the offset ridge segments is not accommodated by a sustained, narrow strike-slip zone. Second-order discontinuities consist of non-transform offsets with distinct off-axis discordant zones. The presence of an off-axis scar suggests that these offsets are persistent. They may migrate with respect to the spreading centre, leaving traces on the flanks of the MAR that are distinct from small circles about the pole of opening of the two plates (Fig. 4). We have identified a spectrum of possible morphological expressions of second-order discontinuities which are controlled primarily by the distance between the two offset spreading segments (Fig. 3a–c). Third-order discontinuities correspond to zero or near-zero, intra-rift offsets of the spreading centre that do not leave a recognizable off-axis signature (Fig. 3d). The absence of an off-axis discordant zone suggests that third-order discontinuities are short-lived features.

Seismic and gravity studies have established that transform faults along the MAR may be associated with crustal thinning^{18,19}. We suggest that non-transform discontinuities are also associated with discontinuities in the structure of the oceanic crust²⁰. Existing seismic data^{21–23} tend to support this hypothesis. Our model assumes that the availability of melt is enhanced beneath the shallowest portion of each segment. Along-strike variations in the availability of melt will result in along-strike crustal-thickness variations at the segment scale. Within our survey area, the complete mantle Bouguer anomalies, corrected for density variations due to the cooling of the lithosphere, exhibit banded gravity lows aligned in the spreading direction. These anomalies are consistent with crustal thickening near the middle of each segment and crustal thinning beneath the spreading-centre discontinuities²⁴.

The behaviour and evolution of transform and non-transform discontinuities may be inferred from their off-axis traces in older

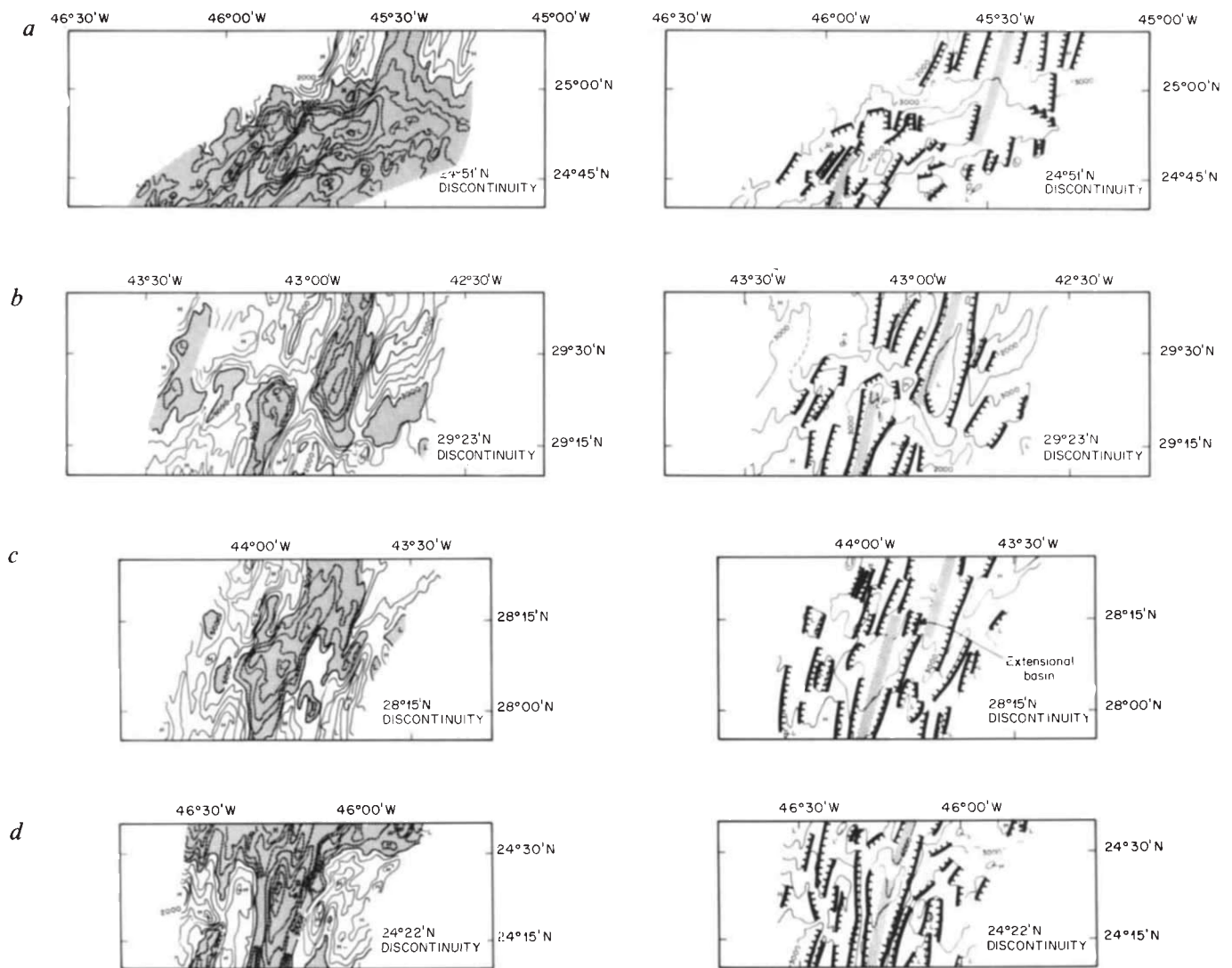


FIG. 3 Bathymetric (left) and schematic tectonic (right) maps of non-transform discontinuities along the MAR (contour interval = 200 m). Areas deeper than 3,000 m are shaded on the left. The location of the neovolcanic zone is indicated by a stippled line on the right. *a*, The 24°51' N discontinuity (offset ~30 km). This is not associated with a clear off-axis trace (which would denote transform motion). The two offset neovolcanic zones terminate near two ill-defined nodal basins; these basins are reminiscent of those found at ridge-transform intersections, but there is no evidence for the presence of a sustained, narrow strike-slip fault zone. *b*, The 29°23' N discontinuity (offset ~17 km). This consists of two offset rift valleys which overlap by 12–15 km. The rift valleys are separated by a 2,400-m-deep high, which rises up to 1,600 m above the valley floors. This discontinuity is associated with an off-axis trace consisting of isolated basins (shaded),

which indicates that it has persisted with time. *c*, The 28°15' N discontinuity. This corresponds to a 9-km offset, which is accommodated by a rectangular, fault-bounded, extensional basin. The continuity of the rift-valley walls indicates that the present offset is recent, but coverage farther off axis suggests that it has also existed in the past. *d*, The 24°22' N discontinuity. This is located at a local along-axis depth maximum, but it does not correspond to a measurable offset of the neovolcanic zone. The neovolcanic zone, which is well defined to the north and south, is ill defined near 24°22' N, suggesting a decrease in the magmatic budget of the spreading centre. This discontinuity may be a true zero-offset discontinuity, in contrast to the term 'zero-offset transform'³⁰, which is often applied to finite-offset discontinuities. There is no noticeable off-axis scar.

sea floor. Like transform offsets, second-order discontinuities are persistent features that leave noticeable scars off axis, consisting of isolated basins that interrupt the rift mountain terrain (Fig. 3). These basins, which are elongated along-axis, may be the fossil equivalents of the marginal lows found at the distal ends of the rift valley within each segment. They integrate into northward-pointing, V-shaped, discordant zones away from existing on-axis discontinuities (Fig. 3). The V-shaped traces point toward the north, as observed in the TAG^{25,26} and MARK²⁷ areas.

The orientation of the off-axis traces of the discontinuities may reflect the relative southward migration of the spreading

centre with respect to an underlying asthenospheric reference frame²⁸ at an average velocity of ~2 mm yr⁻¹ (Fig. 4). The offsets are migrating 'uphill' with respect to the long-wavelength depth variations of the MAR. There is a good general agreement between our interpretation based on our limited off-axis bathymetric coverage and the traces left by the discontinuities as evidenced in the more regional perspective provided by the Seasat-derived gravity field.

Along the fast-spreading EPR, second- and third-order discontinuities correspond to large- and small-offset overlapping spreading centres¹⁴. The morphology of these discontinuities is different from that of the non-transform offsets within our survey

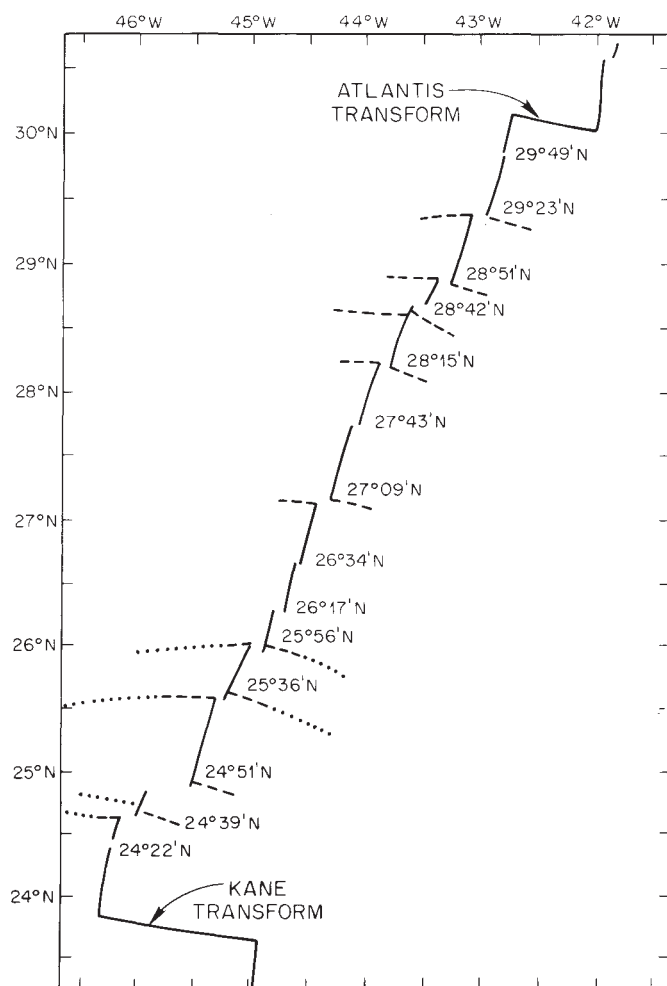


FIG. 4 Off-axis discordant zones associated with non-transform discontinuities between the Kane and Atlantis Transforms. Thick bold lines indicate spreading segments; dashed lines mark the off-axis traces, apparent in our bathymetric coverage, of the non-transform discontinuities; dotted lines represent the off-axis trace of the discontinuities located between 24°N and 27°N, based on narrow-beam bathymetry^{25,26}. The orientation of the off-axis traces is consistent with the southward migration of the spreading centre with respect to an underlying, asthenospheric frame of reference²⁸.

area (Fig. 3). Non-transform discontinuities along the EPR are unstable and may migrate along the plate boundary. But in contrast to the section of the MAR studied here, they do not all migrate in the same direction or at the same rate along the spreading centre^{14,29}. Despite these first-order differences, our data indicate that the processes by which non-transform discontinuities arise may be similar at low- and fast-spreading rates.

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Segmentation in the chick embryo hindbrain is defined by cell lineage restrictions

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IN the chick embryo hindbrain, morphological segmentation into rhombomeres is matched by metameric patterns of early neuronal differentiation and axonogenesis¹. Boundaries between rhombomeres coincide with boundaries of expression of murine regulatory genes^{2–4}. By clonal analysis using intracellular marking, we show here that the rhombomere boundaries are partitions across which cells do not move. When a parent cell is marked before the appearance of rhombomere boundaries, the resulting clone is able to spread into the neighbouring rhombomere. When marked after boundary appearance, the clone still expands freely within the rhombomere of origin, but it is now restricted at the boundaries. Rhombomeres in the chick embryo thus behave like polyclonal units, raising the possibility that they are analogous to the compartments of insects.

During the second and third days of chick development, the hindbrain is subdivided into a series of metameric units, or rhombomeres, which are conspicuous as periodic undulations of the neural epithelium on either side of the unsegmented floor plate. The earliest neuronal organization of the hindbrain is segmental: neurogenesis of both reticular and branchial motor systems begins with an alternate repeat, neurons in the even-numbered rhombomeres differentiating before those in the odd-numbered rhombomeres; cranial nerve motor nuclei are derived from adjoining pairs of rhombomeres¹.

The matching of the rhombomere pattern with an underlying cellular organization and gene expression pattern indicates that segmentation could be important in the construction of the hindbrain. If so, it is possible that rhombomeres are developmental compartments; cell fates might be decided early in development, in relatively small groups, and mixing between the descendants of adjoining groups would be prevented by lineage restriction at the rhombomere boundaries. Compartments are characteristic of insects, where particular regions of

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TABLE 1 Number of clones respecting or crossing rhombomere boundaries

	Boundary respecting	Boundary crossing	χ^2 (d.f. = 2)	P
All clones	68	13	54.8	<0.000001
Clones marked before boundary formation	38	13	20.4	<0.0001
Clones marked after boundary formation	30	0	39.2	<0.00001

The expected frequencies of crossing (0.4), respecting (0.3) and failing to meet (0.3) the rhombomere boundaries by chance are calculated given the average spatial extent of the clones (55% of rostro-caudal length of a rhombomere), and assuming an error of $\pm 7.5\%$ (of rhombomere length) in determining the spatial position of boundaries. This error is estimated as follows. Most boundaries can be located within one cell diameter, whereas the least distinct, or those that slope away from the optical axis (such as in Fig. 2b, left), can be located to within two cell diameters; at stage 18/19, a rhombomere has an average rostro-caudal length of 27 cell diameters (error range, $\pm 3.75\%$ – 7.5%). A chi-square analysis of the observed frequencies of crossing and respecting the boundaries compared with those expected, rejects the null hypothesis ($P < 0.0001$, 2 degrees of freedom, in each category). By contrast, the fraction of clones failing to meet a rhombomere boundary is slightly, but not significantly, smaller than that expected by chance (observed 0.23, expected 0.3; $P > 0.1$ by chi-square analysis, 1 degree of freedom). The frequency is presumably lower than expected because some of the injections were intentionally targeted to the vicinity of developing boundaries.

the integument are made by the descendants of a small set of founder cells, designated at an early stage and prevented from mixing^{5–8}.

To investigate this possibility, we have undertaken a lineage analysis in the chick embryo hindbrain, marking single neural epithelial cells and visualizing their descendants later in development (Fig. 1). Cells were marked by iontophoresis with a vital fluorescent dye (lysinated rhodamine dextran, LRD) whose molecular size is large enough for it to be passed exclusively to descendants and not to clonally unrelated neighbours by means of gap junctions⁹. Although the dye dilutes with cell division and growth, it is still possible to identify labelled descendants after as many as eight symmetrical divisions, making it a reliable

FIG. 1 Outlines of clones traced from the monitor screen are superimposed on a scale diagram of the stage-19 chick hindbrain (centre) seen from its pial (ventral) aspect. The diagram shows a hindbrain after its roof plate has been removed and the dorsal margin of the alar plates have been deflected to appear as the lateral margins¹. On the left side of the hindbrain (A) are clones descended from single cells marked before the appearance of rhombomere boundaries (e.g. at 5 somites, far left; for times of boundary appearance see Fig. 3); some clones (stipple) respect boundaries, whereas others (black) occupy two adjoining rhombomeres, appearing to have crossed the intervening boundary. On the right side (B) are clones descended from single cells marked after boundary appearance (for example, at 13 somites, far right); all clones respect boundaries and never cross them. Clones descended from marked basal plate cells spread into the floor plate at both A and B ages. Clones that are exclusively floor plate show no evidence of transverse restriction boundaries, and sometimes extend over a rostro-caudal distance exceeding that of one rhombomere (not shown). The four clones drawn in r6 were observed in r2 and r4 and have been drawn here to avoid an overlapping display. AP, Alar plate; BP, basal plate; FP, floor plate; M, midbrain; LRD, lysinated rhodamine dextran.

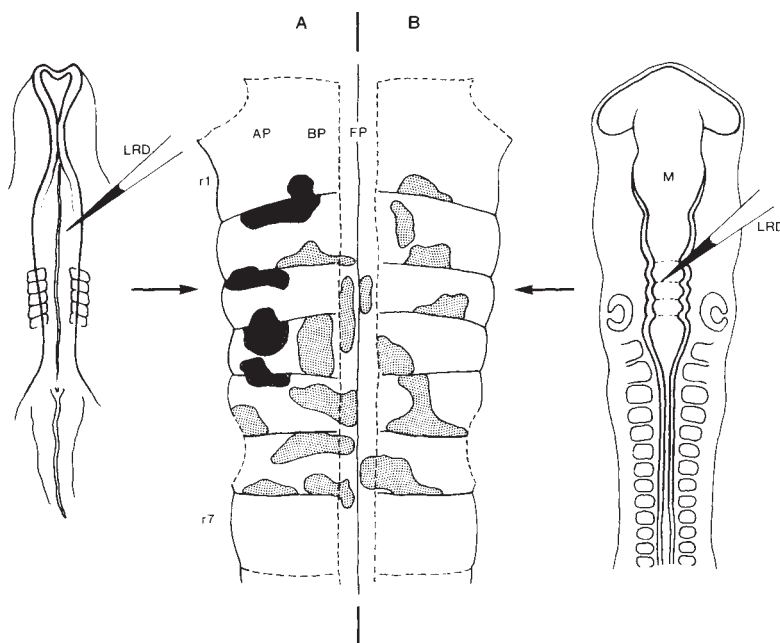
METHODS. Eggs were windowed at 24–48 h of incubation, embryos were made visible by sub-blastodermal injection of India ink and the vitelline membrane was reflected over a small area of the hindbrain. Thin-walled aluminosilicate micro-electrodes (resistance 25–40 megohms) were tip-filled with LRD (100 mg ml⁻¹; Molecular Probes, D-1817) and back-filled with 1.2 M LiCl. The electrode was positioned under bright field epi-illumination, advanced until a surface potential was recorded, and impaled into a cell by briefly ringing the microelectrode with the negative capacitance control. The dye was injected with positive current pulses (250 ms, 4 nA) at 2 Hz for up to 30 s; confirmation that a single cell had been successfully filled was made by direct observation under eipfluorescence. Dye injections were made

and unambiguous lineage marker^{10–12}. We marked a cell in each side of the developing hindbrain, or in its floor plate, at ages ranging between 0 somites (neural plate, stage 6 (ref. 13)) and 15 somites (early neuronal differentiation, stage 12– (ref. 1)) that is, before, during and after the first appearance of the rhombomere boundaries (ref. 14, and Fig. 3). We assayed the distribution of labelled cells after incubation to stage 18–19, when segmentation becomes less pronounced but rhombomere boundaries are still distinct.

Individual LRD-labelled clones in alar and basal plates ($n = 104$) contained between 4 and 64 cells (mean, 16.3 ± 0.8 s.e.m.), and extended $55\% \pm 3\%$ (mean, $\pm$ s.e.m.) of the rostrocaudal length and $53\% \pm 3\%$ of the medio-lateral width of a rhombomere. The labelled, clonally related cells were rarely contiguous, but they were mixed with unlabelled cells in a ratio of 1:3 in the most compact clones, and in a ratio of 1:30 in the most widely dispersed clones (mean, $\sim 1:10$).

We examined the spatial distribution of clones in relation to rhombomere boundaries and noted a marked preponderance of clones ($n = 68/104$) that abutted but did not cross rhombomere boundaries. These clones were often D-shaped, with the flat surface on the boundary, and showed a tendency to expand medio-laterally along the line of the boundary ($n = 63$; Figs 1; 2 a, b, and f; 3). A few other clones ($n = 5$) spanned the entire rhombomere, with flat ends abutting and respecting both rostral and caudal boundaries (Fig. 1). By contrast, we observed a smaller number of clones ($n = 13/104$; Figs 1; 2 c, d and e; 3) in which there were labelled cells on both sides of a boundary, the clone having apparently crossed the boundary to spread in the adjoining rhombomere. Most of the clones meeting a boundary, however, also respect it; the probability that this distribution could arise by chance is less than 0.000001 (Table 1).

Boundary-respecting clones are generated by cells marked at all stages of development, both before the boundary becomes visible and afterwards (Figs 1 and 3). Once the boundaries have become visible, the marking injection can be targeted to a cell lying at or close to a boundary (Fig. 1). All clones developing



into one cell on each side of the hindbrain. After incubation to stage 18–19, embryos were fixed in 4% paraformaldehyde for 2 h, the hindbrain removed and prepared as a flat wholemount in buffered glycerine, and observed from its ventral (pial) aspect under 514–550 nm (green) excitation on a microscope equipped with an image intensifying camera, or with a BioRad MRC 500 laser-scanning confocal microscope.

from such late injections respect the adjacent boundary (Table 1). By contrast, the clones occupying two rhombomeres are, without exception, descended from cells marked before the appearance of the intervening boundary.

The dispersal of labelled cells in a marked clone, and their intermingling with (unlabelled) cells from other clones, shows that individual cells are capable of considerable movement within the sheet of the neural epithelium. Despite the potential for such movement, however, cells do not move across rhom-

bomere boundaries. The existence of boundary-crossing clones does not contradict this finding. In these clones, cells presumably do not literally cross the boundary; rather, one or more labelled daughter cells might move into the territory of the presumptive adjoining rhombomere before the boundary forms. We conclude that rhombomeres are units of cell-lineage restriction.

The existence of clones that respect boundaries and are marked before the appearance of the boundaries indicates that the restriction could precede boundary formation. Alternatively,

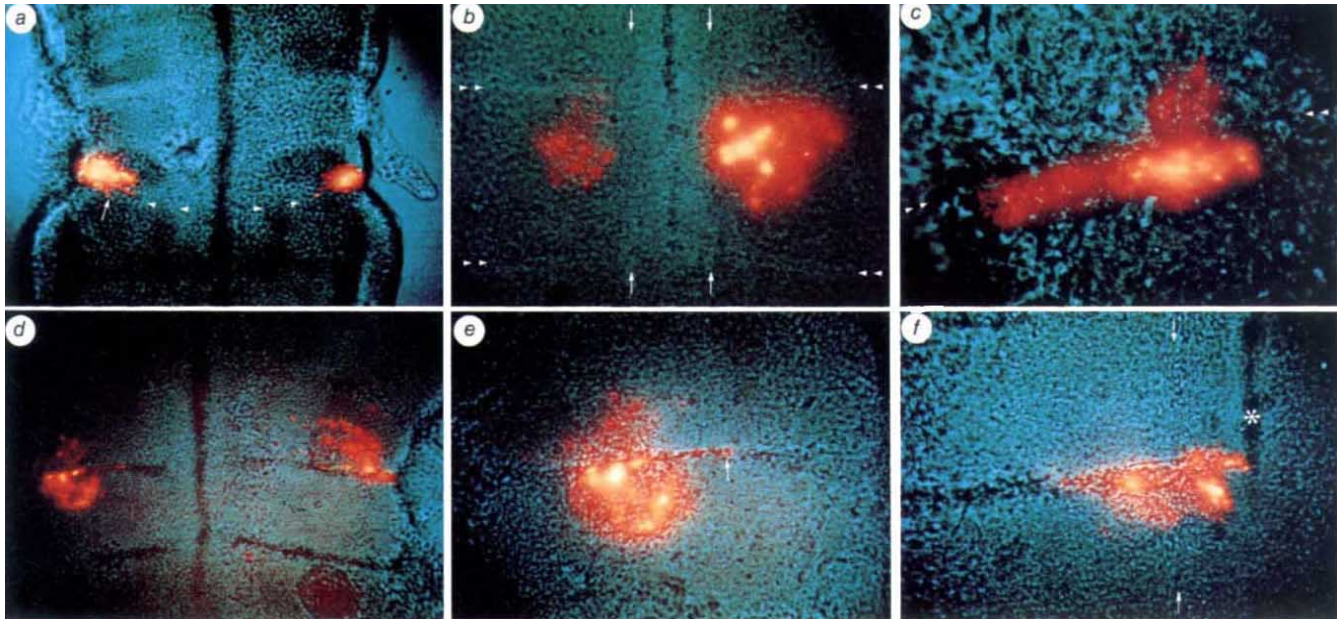
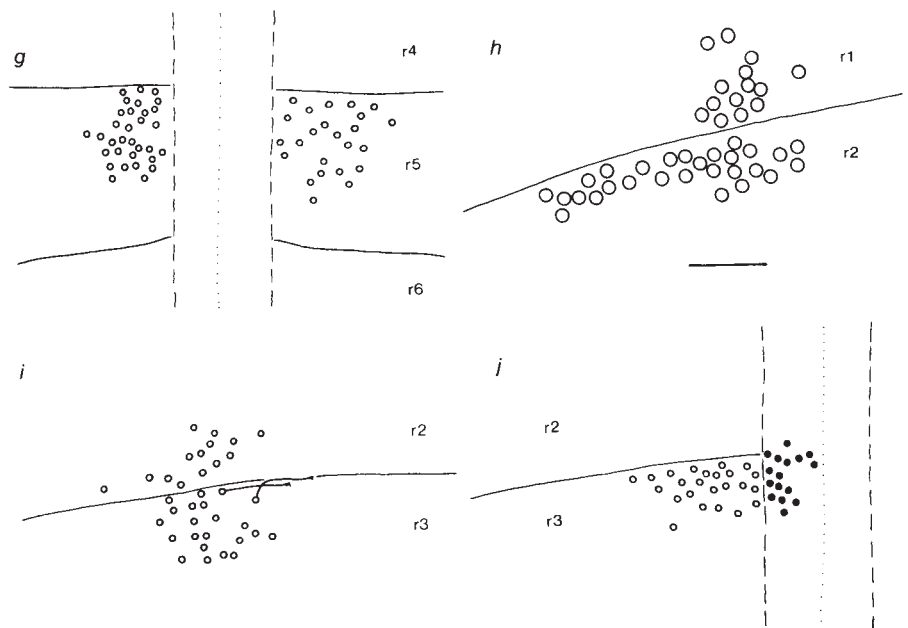


FIG. 2 *a-f*, Combined fluorescence/phase-contrast photomicrographs of whole-mounted hindbrains (prepared as described in the legend to Fig. 1) showing labelled clones descended from single cells marked with LRD. The clone in *c* is also shown in Fig. 1. Rostral is to the top of the panel. *g-j*, Outline tracings of medium- and high-magnification images (*b, c, e* and *f*), each derived from a through-focus series to show the positions of individual epithelial cell bodies in the lateral plate (○) and floor plate (●). Neurons with fluorescent axons are also shown. Rhombomere boundaries (the centres of rhombomere border zones) are shown by solid lines, the basal plate–floor plate boundaries by dashed lines, and the midline by a dotted line. *a*, Clones in the alar plate of rhombomere 5 (*r5*); one cell was injected on each side of the brain at the 1-somite stage of development. Both clones respect the *r5/6* boundary (arrow heads); an axon (arrow) extends from the left side clone. *b(g)*, Clones in the basal plate of *r5*; one cell was injected on each side at 8 somites. Both clones respect the *r4/5* boundary (arrow heads) and the right side clone (24 cells) appears to respect the floor plate/basal plate boundary (arrows). The left side clone contains 32 cells. The *r5/6* boundary is also marked by arrow heads. *c(h)*, Clone in the alar plate of *r2*; the parent cell was injected at 0 somites. The clone is elongate in the transverse axis and a large part of it (28 cells) respects the *r1/2* boundary (arrowheads). At its medial end, the clone extends into *r1* (14 cells); presumably, the *r1/2* boundary formed at an early stage in the formation of this clone, splitting it into two (shown also in Fig. 1). *d*, Clones in the alar plate of *r2* (right) and of both *r3* and *r2* (left); one cell was injected on each side at 5 somites. The right clones respects the *r2/3* boundary, the left clone lies in both *r3* (21 cells) and *r2* (14 cells). Both clones have produced neurons with medially directed axons. *e (i)*, Higher-power view of *d*, left. Medially directed axons (arrow) have grown along the *r2/3* boundary. *f (j)*, Clone at the level of *r3*; parent cell injected at 10 somites. The clone is extended in the transverse axis up to the midline (asterisk); it respects



the *r2/3* boundary but not the FP/BP boundary (arrows). There are 24 cells in the basal plate and 16 in the floor plate. Projections of the *X-Z* plane using the confocal microscope confirm that the labelled floor plate cells have the form of typical radial epithelial cells.

METHODS. The fluorescence image was colour-coded red and the phase image, blue-grey; the coloured images were concurrently displayed using an image processor and photographed from the video monitor (Imaging Technology 151). Line drawings were traced from the video monitor by combining computer-enhanced images of serial focus planes. Scale bar, 160 μm for *a, d*; 80 μm for *b, e, f, g, i* and *j*; 40 μm for *c* and *h*.

it may require several rounds of division for the descendants of an arbitrarily positioned parent cell to reach a position where, after this lapse of time, a boundary has formed. When we targeted injections to the precise axial level of the presumptive r3/4 boundary immediately before its appearance, we found that several clones occupied both r3 and r4 (Fig. 3). We suggest, therefore, that the restriction to cell movement coincides with boundary formation and overt segmentation.

As with the clones described, some clones lie between the rostral and caudal boundaries of a rhombomere but do not meet either ($n = 23/104$; Fig. 1 and Table 1 legend). Cells of these clones, and of others in central regions of the rhombomeres, show no tendency to align or disperse mediolaterally. This indicates that the hindbrain is probably not further sub-partitioned by a set of occult transverse restriction boundaries alternating with the rhombomere boundaries, at least during the period we have studied.

The restriction that occurs at rhombomere boundaries contrasts with the behaviour of clones at another boundary in the

hindbrain, that between the basal plate and the median floor plate (Fig. 1). This boundary is visible in the live embryo from the earliest stage of parent-cell marking, immediately before the appearance of the first somite. We analysed 70 clones in the region of the floor plate–basal plate boundary; of the 42 clones that meet the boundary, 24 respect it and 18 cross it (Figs 2f and 3). Clones from both sides of the boundary respect it, but they cross it only when the parent cell lies in the basal plate. Clones descended from floor plate parents are confined to the floor plate and, with only one exception, are unilateral. These clones contain between 2 and 40 cells (mean, 10.1 ± 0.9 s.e.m) and expand predominantly along the rostro-caudal axis.

The extensive rostro-caudal spread of clonally related cells within the floor plate shows that the restrictions seen more laterally between the rhombomeres do not prevail here. This correlates with an absence of morphological segmentation in the floor plate epithelium¹. At the junction between floor and basal plates, a large proportion of clones cross the already formed boundary, showing that it is not a restriction boundary. This implies that the lineage restriction for individual rhombomeres is incomplete medially; in principle, cells could move from one rhombomere to another by way of the floor plate. But cells seem to cross the boundary in a lateral to medial direction only, basal plate cells becoming floor plate cells, but not vice versa. The fact that a precursor cell in the basal plate can give rise to floor plate cells indicates that the particular characteristics of the floor plate^{15–17} cannot be ascribed to a unique lineage¹⁸, at least at the hindbrain level of the neuraxis. The observation that floor plate clones are almost always unilateral predicts the existence of a midline restriction in this structure.

The cellular and molecular mechanisms whereby partitioning is effected at the boundaries are unknown. One possibility is that rhombomere cells share specific surface properties that render them immiscible with those of adjoining rhombomeres⁷. Another possibility is that specialized cells at boundaries form a mechanical barrier to cell mixing¹⁹. A third possibility is that mutual recognition between adjoining cell groups results in the formation an intervening mechanical barrier. Recent investigations (ref. 1, and S. Guthrie, M. Butcher, R.K. and A.L., manuscript in preparation) reveal several distinguishing features of the rhombomere boundaries, most of which delineate a border zone of ~4–10 cell diameters (see Fig. 2 of ref. 1). But the observation that boundary-respecting clones consistently spread up to the centre of this border zone (Fig. 2g–j) indicates that none of the properties identified so far contributes directly to the restriction.

Compartments in insects contain all the descendants of sets of founder cells whose development is directed by specific regulatory genes⁵. Homologous genes are deployed during the regionalization of vertebrate embryos^{20,21}. Recent studies of a zinc-finger gene² and of certain mouse homoeobox genes^{3,4} have shown, moreover, that their boundaries of expression are aligned with rhombomere boundaries. Like the insect epidermis, therefore, the developing vertebrate hindbrain may use lineage-restricted compartments as units for further pattern formation.

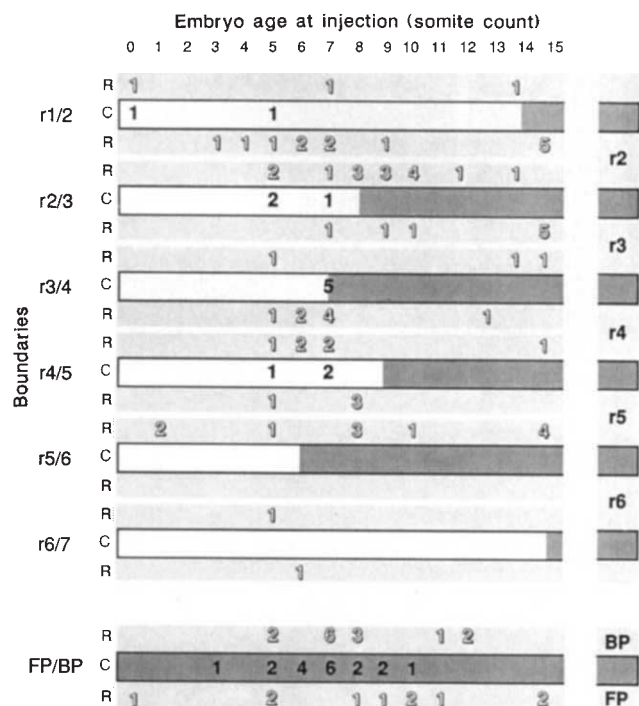


FIG. 3 Chart displaying the numbers of clones marked at progressive somite stages (embryo age at injection, one somite is added every 100 min) and analysed at stage 18–19 (survival time equivalent to ~4–6 cell divisions, assuming a neural epithelial cell cycle time of ~6 h²² at early somite stages, lengthening progressively thereafter). Clone position is shown with respect to the rhombomeres (r2–r6, right) and rhombomere boundaries (r1/2–r6/7, left). The lower part of the figure displays the behaviour of clones at the floor plate–basal plate boundary (FP/BP). The horizontal bars represent the presumptive (white) and definitive (heavy stipple) boundaries in each case. Thus, the r3/4 boundary is first visible at 7 somites and the FP/BP boundary is already visible at 0 somites. Each bar contains the numbers of clones, at each age of marking, that appear to cross (C, left) that boundary (so five clones lie on both sides of the r3/4 boundary when marked at 7 somites; 1 clone crosses the FP/BP boundary when marked at 10 somites). Above and below each C bar, in the lightly stippled areas (R, left), are the numbers of clones that respect the boundary. At all ages of injection, most clones do not cross an adjacent boundary, either from the anterior (above the bar) or from the posterior (below the bar). All rhombomere clones that have apparently crossed a boundary are descended from cells injected before the appearance of the boundary (that is, all crossing clones are within the white region of the C bar, none in the stippled region). At the basal plate–floor plate boundary, clones either cross (C) or respect (R), regardless of embryo age at injection and of the prior appearance of this boundary (so there are crossing clones within the stippled region of the C bar).

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New type of POU domain in germ line-specific protein Oct-4

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MEMBERS of a family of murine octamer-binding proteins interact specifically with the octamer motif, a transcription regulatory element found in the promoter and enhancer regions of many genes¹. Oct-4 is a maternally expressed protein that is also present in the pre-implantation mouse embryo^{1,2}. Although many regulatory proteins are expressed in post-implantation embryos³, transcription factors regulating pre-implantation processes have remained elusive. The Oct-4 gene is therefore a prime candidate for an early developmental control gene. Here we report the complementary DNA cloning of the mouse Oct-4 gene, and the characterization of the encoded protein(s) by sequential *in vitro* transcription, translation, DNA-binding and protease-clipping analysis. Deletion analysis shows that the DNA-binding activity is mediated by a POU domain encoded in an open reading frame corresponding to a 324-amino-acid protein^{4–13}. Sequence comparison with known POU domains reveals that Oct-4 is a novel member of the POU-family.

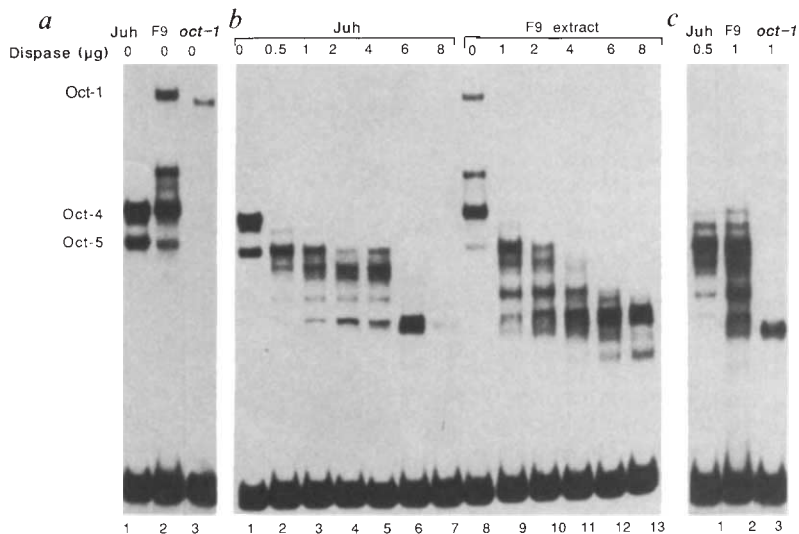
The proteins Oct-1 and Oct-2 both contain a POU domain^{6–10} involved in DNA-binding at octamer motifs¹⁴. Because Oct-4

FIG. 1 Identification of the F9 Oct-4 cDNA clone by EMSA and proteolytic clipping. **a**, Comparison of the juhachi clone (Juh; lane 1) and a mouse Oct-1 clone (lane 3) with F9 octamer-binding proteins (lane 2). Translation products of one group of clones (results for only one are shown) formed a complex migrating slightly ahead of the mouse Oct-1 complex (compare lanes 2 and 3). These clones were identified by sequence analyses as mouse homologues of, and having the same 5' ends as, the human *oct-1* gene⁷, indicating that the difference in migration is probably due to post-translational modifications. A second group of clones yielded DNA-binding products either at the position of Oct-4 and Oct-5 (Juh; lane 1) or smaller (data not shown). The smaller products were identified as 5' deleted juhachi clones. A third group of clones not related to these groups was investigated separately (data not shown). **b**, Proteolytic clipping EMSA of the gene products of the juhachi clone (lanes 1–7) and F9 octamer-binding proteins (lanes 8–13). The proteins were treated without (lanes 1 and 8) or with different amounts of dispase as indicated. **c**, Comparison of octamer-binding intermediates obtained by proteolytic clipping of the translation products of the juhachi clone (lane 1) and an Oct-1 clone (lane 3) with proteolytically degraded octamer-binding proteins of F9 cells (lane 2). Lanes 1 and 2 represent longer exposures of lanes 2 and 9 in **b**, respectively. **METHODS.** Clone 18 (juhachi clone) and clone 3 (Oct-1 clone) were linearized at the unique *XhoI* restriction sites (added at the 3' end of the cDNAs because of the directional cDNA cloning procedure) and purified with Biogel P30 (Biorad) spin-columns. RNA was synthesized with T3 RNA polymerase (Promega) using 50 U μg^{-1} DNA. Reaction conditions, 40 mM Tris-HCl buffer, pH 8.0, 8 mM MgCl₂, 25 mM NaCl, 2 mM spermidine, 5 mM DTT, 2 mM ATP, 2 mM GTP, 2 mM UTP, 2 mM CTP and 2 mM m⁷G(5')ppp5'G. An aliquot of the

binds to the same motif as do Oct-1 and Oct-2, we used a probe spanning the POU domain of the mouse Oct-2 gene to screen a cDNA library prepared from F9 stem cells which contain Oct-4 in abundance (see Fig. 2 legend)^{1,15}. The inserts of 18 positive recombinant phages were transcribed *in vitro* and then translated in a reticulocyte lysate. The translation products were tested for DNA-binding in an electrophoretic mobility shift assay (EMSA) using an oligonucleotide probe containing the octamer motif (probe 1W; Fig. 1).

The products of one clone (which we will refer to as the juhachi clone) shifted the 1W probe to the position of Oct-4 and Oct-5, as found in F9 nuclear extracts (Fig. 1a, lanes 1 and 2). We performed proteolytic clipping experiments to test the relationship of the juhachi-clone products with Oct-4^{1,16}. Reticulocyte extracts containing both the translation products of either the juhachi clone or an Oct-1 clone (see Fig. 1a) and a nuclear extract of F9 cells, were incubated with probe 1W and then digested with different amounts of a nonspecific endoprotease (Fig. 1b, Oct-1 clipping not shown). A comparison of the proteolytic intermediates showed that the pattern with the juhachi-clone gene products is identical to that of Oct-4, with the exception of minor bands most likely resulting from Oct-1 degradation in the F9 extract (Fig. 1b, c). The degradation pattern obtained with the F9 extract seems to be a combination of those of the cloned Oct-1 and Oct-4 proteins (Fig. 1c). The position of the juhachi-clone translation products in the EMSA, and the degradation pattern, strongly indicate that the juhachi clone encodes Oct-4.

Sequence analysis of the cDNA identifies an open reading frame encoding 324-amino-acid protein (Fig. 2a). Six clones yielding smaller octamer-binding proteins (see legend of Fig. 1a) represent incomplete 5' ends of the juhachi clone (clones 1 and 16 of Fig. 2a). Comparison of the sequence of Oct-4 with the sequences of POU domain-containing proteins^{4–13,17} reveals a 79-amino-acid region that is homologous to the POU-specific domain, and a 60-amino-acid region homologous to the POU homoeodomain (Fig. 2a, c). The two regions are separated by a stretch of 17 amino acids which has no homology to any other POU domain-containing protein. Similar to other homoeodomains, that of Oct-4 is predicted to contain four helices, with a cluster of basic amino acids preceding the first helix (cluster I) and a cluster overlapping the end of the third



transcription mixture containing about 1 μg RNA was added to a rabbit reticulocyte lysate (BRL) in accordance with the manufacturer's specifications. The translation products were analysed in parallel by SDS-PAGE (data not shown) and tested for binding in an EMSA. Translation assay (1 μl) was used for the EMSA as described¹, except the amount of poly d(I-C) was reduced to 0.4 μg . The F9 nuclear extract was prepared as described¹. The EMSA was performed with probe 1W containing the octamer of the enhancer of the mouse immunoglobulin heavy chain gene¹.

METHODS. Cloning: Total RNA was isolated from F9 stem cells by the guanidinium thiocyanate method²⁸. Poly(A)⁺ RNAs were prepared by retention on oligo(dT) columns. A directional cloned cDNA library in the λ expression vector λ ZAPII (Stratagene) was constructed essentially as described²⁹ with the following modifications: single-stranded cDNA primed with the oligonucleotide 5'-AATTCCTCGAG(T)₁₅-3' containing a *Xho*I restriction site, was prepared using avian myeloblastosis virus (AMV) reverse transcriptase (Boehringer) and 5-methylcytosine. Double-stranded cDNAs, methylated at internal *Eco*RI sites was ligated to 12mer *Eco*RI linkers (CCGAATTCGG, Boehringer). After recutting with *Eco*RI and *Xho*I, and size fractionation on a Biogel A50m column (Biorad), the cDNA inserts were ligated to *Eco*RI/*Xho*I-digested λ ZAPII vector DNA. After *in vitro* packaging, the phages were plated on *E. coli* PLK-F' (Stratagene). The average insert size was 2.1 kb (0.7–7.6 kb, based on 34 phage 'minipreps'). Screening: for isolation of λ phage recombinants containing POU-related sequences, 10⁶ independent plaques were screened using Hybond membranes (Amersham). The DNA membranes were baked for 2 h at 80 °C. Prehybridization was at 60 °C in 7 $\times$ SSC, 5 $\times$ Denhardt's solution, 0.5% SDS, 0.1 mg ml⁻¹ denatured salmon sperm DNA; hybridization was performed in the same solution containing 10⁶ c.p.m. ml⁻¹ of a 0.6-kb mouse *Oct-2* probe spanning the POU domain (A. Hatzopoulos, unpublished results) at 65 °C for 16 h. The filters were washed in 1 $\times$ SSC, 0.1% SDS, at 25 °C. The inserts were then excised by a helper phage to generate Bluescript II SK recombinant phagemids for sequencing and *in vitro* transcription. Sequencing was performed with the dideoxy chain termination method using single-stranded and double-stranded DNA. (Sequenase Kit, USB). For northern-blot analysis, poly(A)⁺ RNA was isolated as described above. About 5 μ g was electrophoresed through a 1% agarose gel in 3.7% formamide and MOPS buffer (20 mM morpholine propane sulphonic acid, 50 mM sodium acetate, 10 mM EDTA, pH 7.0). After electrophoresis, the gel was soaked in 20 $\times$ SSC for 30 min and blotted onto Gene-Screen-plus (NEN-Dupont) overnight with 10 $\times$ SSC. The filter was baked at 80 °C for 2 h and hybridized overnight with a *Pst*I–*Pst*I *Oct-4* probe (10⁶ c.p.m. ml⁻¹) at 42 °C in 50% formamide, 1 M NaCl, 1% SDS and 100 μ g ml⁻¹ denatured salmon sperm DNA. The blot was washed twice for 15 min in 2 $\times$ SSC, 1% SDS, and then washed in 0.1 $\times$ SSC, 1% SDS at 60 °C. The autoradiograph was exposed overnight.



helix (cluster II)^{18,19} and most of the fourth helix²⁰. Another region rich in basic amino acids is located in the centre of subdomain A of the POU-specific domain (cluster III). The regions outside the POU domain are rich in proline residues, interspersed with acidic amino acids at the N-terminal region of the protein. Transcriptional activation by this acidic region, as has been described for other proteins²¹, remains to be shown.

The POU domain of Oct-4 is different from all classes of POU domains described so far¹¹; it has the highest homology to class III POU domains (Fig. 2d). Two regions of homology between the POU domain of Oct-4 and class III POU domains are especially striking: subdomain B of the POU-specific domain¹⁷, and the possible hinge region between helices 1 and 2 of the POU homeodomain²⁰. Subdomain B is identical at 30 of 34 positions to rat SCIP (Tst-1)^{11,13}, and at 31 positions to the *Caenorhabditis elegans* protein Ceh-6 (ref. 12). Moreover, two of the remaining amino acids represent conservative changes (Leu-Val; Lys-Arg). In the hinge region, 100% identity to SCIP (Tst-1) is found, although the flanking helices show only about 40% homology. This homology could reflect an interaction of SCIP (Tst-1) and Oct-4 with a common target. It is interesting that although SCIP (Tst-1) and Ceh-6 are highly homologous members of class III POU domain-containing proteins their homology in the hinge region is only 37%. The highest similarity between all classes and Oct-4 is found in the predicted recognition helix (helix 3). Only one substantial change in this helix is found between Oct-4 and the members of class IV proteins.

A 1.6-kilobase (kb) transcript was detected in F9 stem-cell RNA using an Oct-4-specific probe (Fig. 1b). No Oct-4 transcript could be detected in RNAs of day-12.5 embryos, testis, liver, lung, intestine, brain or kidney (results not shown). This is support for the expression of Oct-4 being strongly developmentally regulated^{1,2}.

The use of two in-frame ATG codons from one transcript to generate different products has been predicted for a variety of

proteins, including some oncogenes, hormone receptors and growth factors²². The two translation products produced by the juhachi-clone RNA (see Fig. 1a, lane 1) could also result from the use of the two ATG codons at the 5' end of this clone. To test this possibility, we deleted the first ATG codon. Translation of the resulting RNA produced a protein that migrated to the Oct-5 position in the EMSA, showing that both codons can be used for initiation (Fig. 3a). Although the similarity in size of the smaller translation product and Oct-5 is highly suggestive, it remains to be demonstrated whether Oct-5 is indeed translated from the Oct-4 RNA *in vivo*. Alternatively, the production of differential splicing products with or without the first AUG could also give rise to both proteins. If such post-transcriptional regulation occurs, the different ratios of Oct-4 to Oct-5 in F9 cells and unfertilized oocytes¹ would then indicate that the expression of these proteins is differentially regulated during mouse development. Indeed, differential expression of translational initiation can be mimicked *in vitro* (Fig. 3b).

We examined the DNA-binding domain by using two of the 5' deletions of the Oct-4 gene and further deletions using suitable restriction sites. Analysis of the different 5' deletions reveals that binding still occurs when the POU-specific domain is nearly deleted. The shorter protein encoded by clone 1, initiating translation at the C-terminal half of the POU subdomain B, can still bind to DNA (Fig. 4, B in a (lane 5) and c). But when the same clones were restricted with *Bgl*I or *Ban*I before *in vitro* translation to remove the C terminus²³⁻²⁵, the clones with lesser 5' deletions encoded proteins that still bound DNA, whereas the protein encoded by the clone with the largest 5' deletion (clone 1) completely lost DNA-binding activity (Fig. 4a, lane 6). This deletion of the C terminus removes three of the five basic amino acids of cluster II (Fig. 4c). Because shortened translation products that retain the complete POU domain form DNA complexes, sequences in the POU-specific domain must contribute to DNA-binding. In addition, because the translation

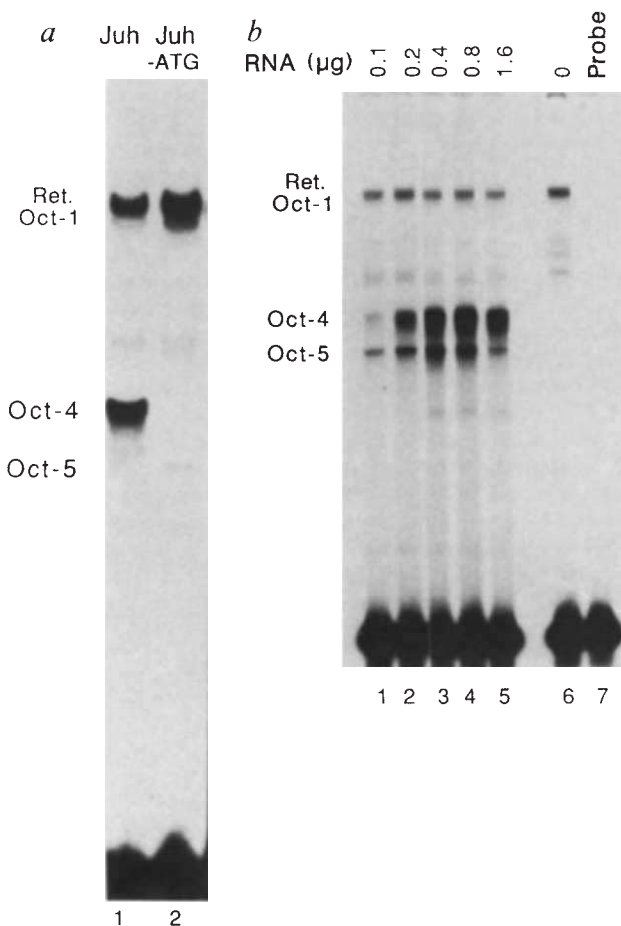
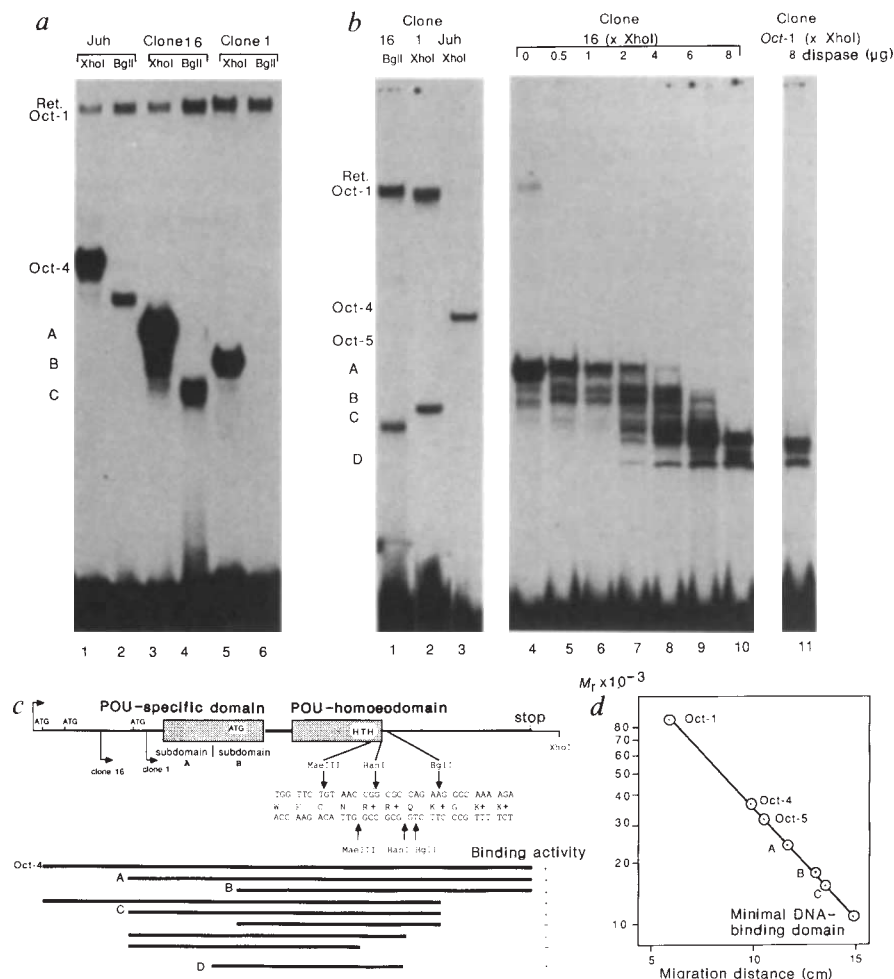


FIG. 3 *In vitro* translation of Oct-4 and Oct-5. *a*, Examination of initiation codon use in the juhachi clone (Juh). The translation products of the juhachi clone (lane 1) and a 5'-deleted clone without the first ATG (lane 2) are compared. The ATG was deleted by cutting the juhachi clone with *Bam*HI and then religating. One *Bam*HI site resides in the polylinker upstream of the gene, the other overlaps with the first ATG codon (Fig. 2a). The positions of Oct-4 and Oct-5 are indicated. *b*, Differential *in vitro* translation of Oct-4 and Oct-5. Different amounts of the *in vitro* transcription assay containing juhachi clone RNA were incubated with the reticulocyte lysate. The amounts of RNA are indicated above lanes 1-5. Lane 6, extract without RNA; lane 7, probe. Ret.Oct-1 is Oct-1 derived from the reticulocyte lysate.

FIG. 4 Delineation of the DNA-binding domain. *a*, Determination of the C-terminal border of Oct-4 DNA-binding domain. The juhachi clone (Juh; lanes 1, 2), clone 16 (lanes 3, 4) and clone 1 (lanes 5, 6) were cut either with *XhoI* (lanes 1, 3, 5), *BglI* (lanes 2, 4, 6), *BanI* (data not shown) or *MaeIII* (data not shown) before expression and EMSA. In parallel, ³⁵S-labelled translation products were analysed on an SDS-PAGE showing slightly greater amounts of the clone-16 translation products (data not shown). *b*, Estimation of the N-terminal border of Oct-4. The translation products produced by restriction of the different clones (lanes 1–4) are shown in parallel with the degradation intermediates of the clone-16 translation product (lanes 4–10) and the final detectable products of an Oct-1 protein (lane 11). To use the translation products as length markers, different amounts of protein were applied to the EMSA. Therefore the intensities of the complexes do not reflect DNA-binding affinity. Ret. Oct-1 in *a* and *b* is Oct-1 derived from the reticulocyte lysate. *c*, Schematic representation of the Oct-4 protein indicating corresponding positions of 5' deletions and restriction sites used for protein mapping. The C-terminal border of the homeodomain is enlarged to show the nucleotide sequence, the deduced amino-acid sequence (single-letter code) and the restriction sites used in *a*. The lengths of the respective proteins and their binding activity are shown below. A–D, translation products of the different clones or their shorter counterparts. *d*, Length calibration of the minimal Oct-4 DNA-binding domain by extrapolation¹⁶ using the M_r s of the products shown in lanes 1–4 of Fig. 4*b* (see text).



product of clone 1 which is missing most of the POU-specific domain can still bind to DNA, sequences C-terminal to the POU homeodomain must also contribute to DNA-binding. Therefore, stable complex formation is possible when at least one of these regions is present.

To determine the minimal DNA-binding domain of Oct-4, the EMSA with the deletion mutants was combined with the proteolytic clipping assay^{1,16}. The *BanI* site marks the C-terminal border of the minimal DNA-binding domain, because any further removal of amino acids abolished stable binding (*MaeIII* restriction in Fig. 4*c*). Moreover, proteolytic clipping of the complexes with the full-length or the shorter translation products of clone 16 and clone 1 gave the same final complexes (Fig. 4*b*, only clone 16 restricted with *XhoI* is shown). To determine the approximate size of the minimal DNA-binding domain, the different translation products were used as markers (Fig. 4*b*, lanes 1–4) and their calculated relative molecular masses (M_r) were plotted against the distances of migration on a logarithmic scale (Fig. 4*d*). The M_r of the minimum binding protein was ~11,000, corresponding to 100 amino acids as deduced by extrapolation. According to this calculation, the N-terminal border would coincide with the border between subdomains A and B of the POU-specific region (Fig. 4*c*).

Oct-4 is present in primordial germ cells, unfertilized oocytes and in the inner cell mass of the mouse blastocyst¹. Therefore, this putative transcription factor could act at the very top of the hypothetical cascade of control events. The studies described here show that Oct-4 contains a new type of POU domain (class V). Although it is necessary for DNA-binding, this domain could also function by interacting with other proteins. Two sequences highly conserved between the POU domain of Oct-4 and class III POU domains are good candidates for further studies. We

believe that our results will help in the study of the function of Oct-4 during murine embryogenesis.

Note added in proof: After this manuscript was submitted, Okamoto *et al.*³⁰ described the cDNA cloning of an octamer-binding protein (Oct-3; from P19 cells) that is probably also encoded by the Oct-4 gene. There are two differences between the P19 and the F9 cDNA clones. First, a difference at the C-terminus that can be explained by an additional G in our sequence at position 800 which results in a frameshift. We do not think that this additional G is due to the cDNA cloning procedure. Without this frameshift Oct-4 and Oct-5 would be 25 amino acids longer. Such a difference would be detectable in the EMSA. However, Oct-4 and Oct-5 expressed from the Oct-4 cDNA clone are at the same position as F9 Oct-4 and Oct-5. Second, the Oct-4 and Oct-3 cDNA clones have different 5' sequences, possibly due to differential splicing. This difference results in a N-terminal replacement of the MetAspPro sequence in Oct-4 by 31 different amino acids in Oct-3. The differences between Oct-4 and Oct-3 do not affect the POU domain. □

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Peptide ligand-induced conformation and surface expression of the L^d class I MHC molecule

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NEWLY synthesized major histocompatibility complex (MHC) class I molecules in the endoplasmic reticulum are thought to bind peptides of foreign and endogenous antigens^{1–4}. Several lines of evidence indicate that β -2 microglobulin (β ₂m) and/or peptide ligand participate in the intracellular transport and surface expression of class I molecules, but the nature of their involvement is still unclear^{5,6}. Here we present evidence that culturing non-mutant cells (fibroblast, thymoma or mastocytoma) with a peptide ligand specific for the L^d class I molecule of the mouse leads to a dramatic (fourfold) and specific induction of L^d surface expression. Surprisingly, this peptide ligand-induced expression of L^d does not result in an increased intracellular association of L^d with β ₂m. These findings demonstrate that the previously reported decrease in surface expression of L^d results from its failure to be saturated with endogenous self-peptide ligands. This unique feature of L^d could also contribute to the fact that several virus-specific cytotoxic T cell responses have been found to be L^d-restricted.

Certain class I molecules such as human HLA-C^{7–9} molecules and the L^d and D^k molecules of the mouse^{10,11}, are distinguished by their weak associations with β ₂m, their slower intracellular transport and/or lower cell-surface expression. Of these class I molecules, the L^d molecule has been most extensively investigated^{11–13}. To test whether β ₂m is the limiting factor controlling the transport and expression of L^d molecules, we produced a cell line in which β ₂m was overexpressed relative to L^d and found no significant increase in L^d transport or surface expression (data not shown). Because this suggested to us that β ₂m was not the limiting factor, we next investigated whether peptide ligand controls L^d expression using peptides derived from the murine cytomegalovirus (MCMV) or the tum[–] P815 tumour variant. These peptides have been shown specifically to use L^d as the restricting element for cytotoxic T lymphocyte (CTL) recognition^{14,15}. We cultured L^d-positive cell lines in medium with or without peptide as described⁶. L–L^d, an L-cell fibroblast line transfected with the L^d gene, was cultured for

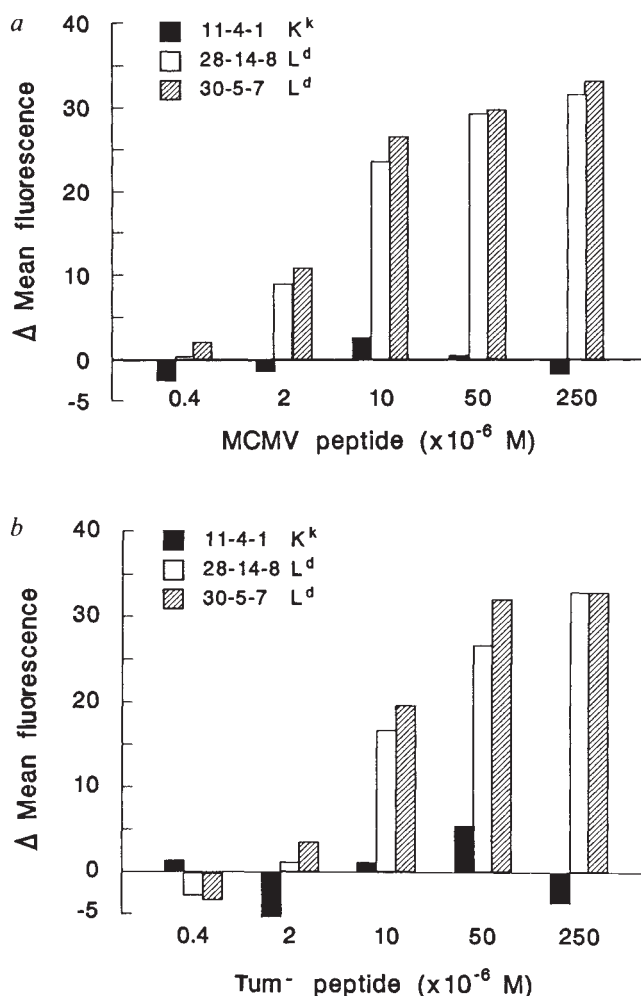


FIG. 1 Specific induction of L^d surface antigens on an L^d gene-transfected L-cell line (L–L^d). **a**, Dose response of L–L^d cells to MCMV peptide. The relevant amino-acid sequence of the MCMV peptide corresponds to residues 168–176 (Tyr-Pro-His-Phe-Met-Pro-Thr-Asn-Leu) of the murine cytomegalovirus (MCMV) immediate-early protein pp89 (ref. 14). **b**, Dose response of L–L^d cells to tum[–] peptide. Amino-acid sequence of the tum[–] peptide corresponds to residues 12–24 (Ile-Ser-Thr-Gln-Asn-His-Arg-Ala-Leu-Asp-Leu-Val-Ala) of the mutant protein P91A[–] (exon 4) from the tum[–] P815 variant¹⁵. A control D^b-restricted peptide, influenza NP 365–380 (ref. 6) did not induce expression of L^d (data not shown). L–L^d cells were exposed to various concentrations of peptide for 15 h and then stained with monoclonal antibody 11-4-1, 28-14-8, or 30-5-7 to detect K^k, L^d (α 3), or L^d (α 1/ α 2) antigens respectively. The results were obtained with logarithmic amplification of fluorescence intensity expressed in channels, where 4 logs span 256 channels (64 channels per log). Our FACS machine has been calibrated such that the relative increase is equal to 10⁰, where $e = \Delta$ mean fluorescence (channels)/64 (channels/decade). According to this formula, an increase in fluorescence intensity of 40 channels represents a 4.2-fold increase in expression and 30 channels represents a 3-fold increase in expression.

METHODS. Peptides MCMV pp89 (168–176) and tum[–], P91A[–] (12–24), were made using an applied Biosystems Model 430A peptide synthesizer and standard t-Boc chemistry. 3.3×10^5 cells per ml were cultured with either medium (DMEM/10% FCS) alone or with various concentrations of peptide for 15 h at 37 °C, 6.5% CO₂. After spinning, cells were resuspended in Hanks balanced salt solution containing 2% BSA and 0.2% sodium azide and analysed by indirect immunofluorescence. A saturating concentration of monoclonal antibody 11-4-1, 28-14-8, or 30-5-7 was used to detect K^k, L^d (α 3) and L^d (α 1/ α 2) antigens respectively^{24,25}. As a developing reagent, we used a saturating concentration of fluorescein-conjugated F(ab')₂ fragment of goat anti-mouse IgG, Fc-specific (Organon Teknika-Cappel). Cells were analysed on a fluorescence-activated cell sorter (FACS IV, Becton Dickinson) and fluorescence histograms generated with logarithmic amplification of fluorescence emitted from single viable cells. Background staining did not vary significantly when treated with peptide or negative control antibody.

TABLE 1 Specific induction of L^d antigens on the surface of various class I gene-transfected cell lines using the MCMV peptide

Cell line	Monoclonal antibody	H-2 reactivity (domain)	Mean fluorescence (channels)*		Difference
			-Peptide	+Peptide	
L-L ^d	28-14-8	L ^d ($\alpha 3$)	75.2	113.2	38.0
	30-5-7	L ^d ($\alpha 1/\alpha 2$)	78.0	113.1	35.1
	11-4-1	K ^k	65.4	66.5	1.1
L-m/L ^d	28-14-8	L ^d ($\alpha 3$)	63.4	104.7	41.3
	30-5-7	L ^d ($\alpha 1/\alpha 2$)	67.9	105.9	38.0
	11-4-1	K ^k	76.9	78.9	2.0
L-m/L ^d - β_2m	28-14-8	L ^d ($\alpha 3$)	74.7	111.0	36.3
	30-5-7	L ^d ($\alpha 1/\alpha 2$)	77.5	113.3	35.8
	11-4-1	K ^k	87.3	85.9	-1.4
L-m/D ^d	34-2-12	D ^d ($\alpha 3$)	108.0	103.2	-4.8
	34-5-8	D ^d ($\alpha 1/\alpha 2$)	107.8	109.3	1.5
	11-4-1	K ^k	77.6	79.4	1.8
L-L ^d L ^d D ^d	34-2-12	D ^d ($\alpha 3$)	39.3	68.9	29.6
	30-5-7	L ^d ($\alpha 1/\alpha 2$)	37.0	65.9	28.9
L-D ^d D ^d L ^d	28-14-8	L ^d ($\alpha 3$)	73.9	69.9	-4.0
	34-5-8	D ^d ($\alpha 1/\alpha 2$)	72.3	72.7	0.4
L-D ^b	28-14-8	D ^b ($\alpha 3$)	104.8	100.4	-4.4
	66	D ^b ($\alpha 1/\alpha 2$)	44.0	46.0	2.0
	11-4-1	K ^k	12.6	10.8	-1.8
L-L ^q	28-14-8	L ^q ($\alpha 3$)	89.0	113.3	24.3
	66-13-5	L ^q ($\alpha 1/\alpha 2$)	74.2	102.3	28.1
	11-4-1	K ^k	22.7	24.7	2.0
R11-L ^d	28-14-8	L ^d ($\alpha 3$)	83.3	103.2	19.9
	30-5-7	L ^d ($\alpha 1/\alpha 2$)	80.6	98.3	17.7
	11-4-1	K ^k	63.8	60.1	-3.7
R11-D ^d	34-2-12	D ^d ($\alpha 3$)	68.6	69.6	1.0
	34-5-8	D ^d ($\alpha 1/\alpha 2$)	67.4	66.6	-0.8
	11-4-1	K ^k	62.5	63.2	0.7

Cell lines L-L^d, L-m/L^d, L-m/D^d, L-D^b, and L-L^q were generated by introducing the L^d gene, the L^d gene with a metallothionein promoter¹³, the D^d gene with a metallothionein promoter, the D^b gene or the L^q gene into thymidine kinase-deficient mouse L-cell fibroblasts (H-2^k) by calcium phosphate co-precipitation. The double transfectant L-m/L^d- β_2m was generated similarly by introducing the β_2m gene into L-m/L^d cells. The exon-shuffled transfectants, L-L^dL^dD^d ($\alpha 1/\alpha 2$ of L^d, $\alpha 3$ of D^d) and L-D^dD^dL^d ($\alpha 1/\alpha 2$ of D^d, $\alpha 3$ of L^d) were gifts from D. Margulies¹⁶. Cell lines R11-L^d and R11-D^d were generated by transfecting the L^d or D^d genes respectively into R11 (H-2^k) thymoma cells by electroporation. Cells were incubated with medium alone (-peptide) or with MCMV peptide at 5×10^{-5} M (+peptide) for 15 h as described in the legend to Fig. 1, and then analysed by indirect immunofluorescence using the monoclonal antibody indicated^{24-27,30}.

* Mean fluorescence above background staining in the presence only of the fluorescent antibody.

15 h with different concentrations of peptide. As shown in Fig. 1, expression of L^d on L-L^d cells increased dramatically and in a dose-dependent way in response to both peptides, as indicated by staining with the monoclonal antibodies 30-5-7 ($\alpha 1/\alpha 2$ domains) or 28-14-8 ($\alpha 3$ domain)¹⁶, whereas the expression of the L cell-derived K^k antigen was unaffected at all peptide concentrations. As shown in Table 1, specific induction of L^d molecules was also seen with L cells transfected with the L^d gene under the control of the metallothionein gene promoter

(L-m/L^d)¹³ and R11 thymoma cells transfected with an L^d gene (R11-L^d). There was no induction of D^d or D^b molecules with the MCMV peptide in transfected L-cell lines (Table 1). Using exon-shuffled constructs¹⁶, peptide-specific induction was mapped to the 5' portion of the L^d gene, a region containing the $\alpha 1$ and $\alpha 2$ ligand-binding domains¹⁷. The MCMV peptide also induced the expression of L^q antigens (Table 1), which differ from L^d by only six amino acids in the $\alpha 2$ domain¹⁸. As shown in Fig. 2, these peptide ligands induced expression of

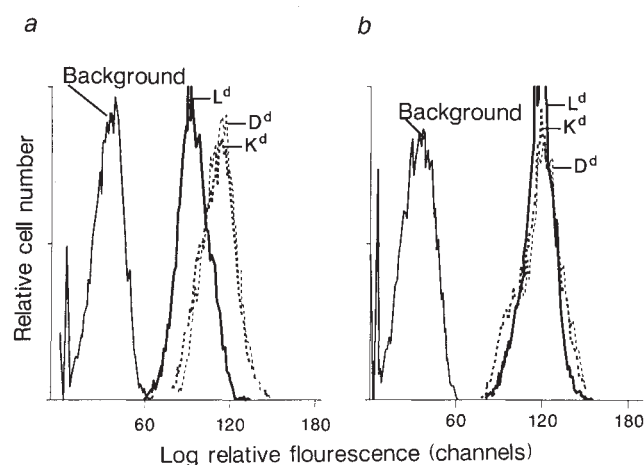


FIG. 2 Peptide treatment of P815 tumour cells specifically increases the expression of L^d antigens compared to D^d and K^d antigens. Cells were incubated *a*, with medium alone or *b*, with MCMV peptide at 5×10^{-5} M for 15 h at 37 °C. The same results were obtained with the tum⁻ peptide, P91A⁻ (refs 12-24) (data not shown). Indirect immunofluorescence methods are described in the legend of Fig. 1. Profiles representing background staining were obtained using only the second antibody; antibodies 28-14-8, 34-2-12 and MA215 were used to detect L^d, D^d and K^d antigens respectively²⁵⁻²⁷. Other antibodies against L^d, D^d and K^d antigens gave the same results (data not shown).

endogenous L^d on P815 (H-2^d) mastocytoma cells to a level comparable to that of D^d and K^d antigens. The increased fluorescence shown in Figs 1 and 2 and in Table 1 represents a maximum fourfold increase, indicating that at least 75% of the total surface L^d molecules should contain the fed peptide. Such a level of homogeneity makes this an ideal system to study the class I-peptide ligand interaction.

The L^d molecule can be detected by sequential immunoprecipitation in two antigenic forms, one that is positive with antibody 30-5-7 and with other antibodies against $\alpha 1/\alpha 2$ epitopes, the other (30-5-7⁻) that can only be detected with antibody 28-14-8 ($\alpha 3$ -specific)¹⁹. The 30-5-7⁺ L^d molecules are found as either free heavy chains or associated with β_2m , whereas the 30-5-7⁻ L^d molecules are not associated with β_2m . To determine the effect of peptide ligand on the alternative forms of L^d , we sequentially immunoprecipitated lysates of cells grown with or without MCMV peptide. After biosynthetic labelling of L-L^d cells (Fig. 3a), control lysates contained a 1:2 ratio of 30-5-7⁺ to 30-5-7⁻ L^d molecules, whereas this ratio was 20:1 in cells grown with MCMV peptide. Significantly, even after peptide induction, intracellular L^d molecules remain predominantly as free heavy chains, confirming that L^d has a very weak avidity

for mouse β_2m ¹¹. Aliquots of 30-5-7⁺ L^d molecules from either control (lane 1) or peptide-fed (lane 4) L-L^d cells were completely sensitive to endoglycosidase H (data not shown). With surface-labelled L-L^d cells (Fig. 3b), control lysates contained a 1:2 ratio of 30-5-7⁺ to 30-5-7⁻ L^d molecules, whereas a ratio greater than 20:1 was found in peptide-fed cells. The surface 30-5-7⁺ L^d molecules show a strong β_2m association, which from previous studies is known to be bovine β_2m ¹⁹. Thus, these immunoprecipitation experiments indicate that incubation with MCMV peptide dramatically and specifically induces 30-5-7⁺ L^d molecules intracellularly and at the cell surface.

We have shown that 30-5-7⁺ L^d molecules are preferentially transported relative to 30-5-7⁻ L^d molecules¹⁹, so the higher proportion of 30-5-7⁺ L^d molecules induced by peptide treatment could explain the increase in surface L^d expression. At least part of this induction of 30-5-7⁺ L^d must occur in a pre-Golgi compartment²⁰ as the effect already noted was observed on L^d molecules sensitive to endoglycosidase H. Thus, we propose that 30-5-7⁻ L^d molecules accumulate intracellularly owing to a lack of endogenous peptides and/or proper folding. Once bound by ligand, L^d molecules become 30-5-7⁺ and are then preferentially transported to the plasma membrane. Such a mechanism is consistent with that proposed by Townsend *et al.*⁶ using peptide to induce D^b or K^b expression on a class I-deficient mutant cell line. But our non-conformed (30-5-7⁻) L^d molecules are expressed on the cell surface without treatment with peptide, whereas in their studies non-conformed (B22/249⁻) D^b molecules are retained by the endoplasmic reticulum⁶.

In conclusion, feeding of specific peptide ligand seems to correct two anomalous features of L^d : the lower cell surface expression, and the detection of non-conformed (30-5-7⁻) molecules. Interestingly, these peptide-induced corrections are accomplished without increasing the weak avidity of L^d for mouse β_2m . By comparison, we have so far not observed appreciable induction of other class I molecules with their known respective ligands on various non-selected cell types (data not shown). Thus, the unique feature of L^d causing its aberrant expression seems to be a defective binding of available endogenous self peptides. This property could help explain why the L^d molecule is the restriction element for CTL recognition of viruses such as vesicular stomatitis virus, lymphochoriomeningitis virus and cytomegalovirus²¹⁻²³. □

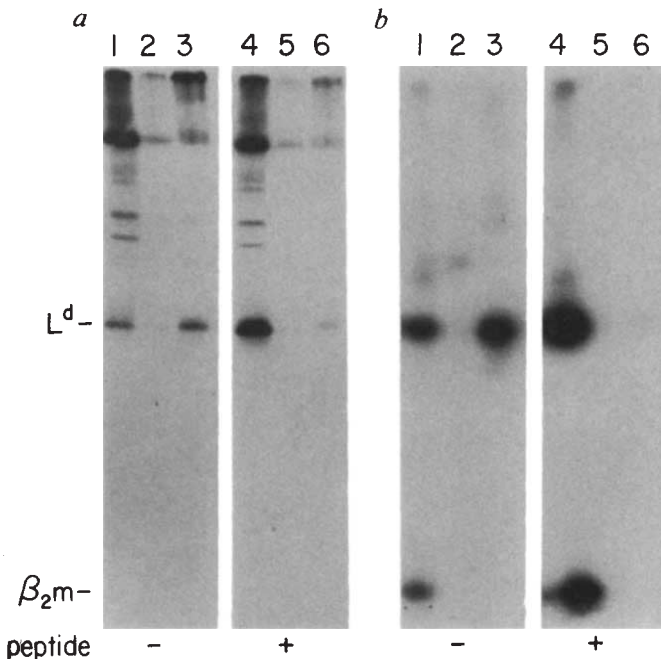


FIG. 3 Comparison of the alternative antigenic forms of L^d from L-L^d cells grown with or without MCMV peptide. Precipitates of L^d from lysates of ³⁵S-labelled cells grown without or with MCMV peptide are shown in a, lanes 1-3 and 4-6 respectively. Similarly, precipitates of L^d from ¹²⁵I-labelled cells grown without or with MCMV peptide are shown in b, lanes 1-3 and 4-6 respectively. Alternative antigenic forms of L^d were resolved from each lysate by sequential immunoprecipitation, treating initially with 30-5-7 (lanes 1 and 4), followed by 30-5-7 to show complete clearance (lanes 2 and 5), and concluding with 28-14-8 to detect 30-5-7⁻ L^d molecules (lanes 3 and 6). Thus, from each lysate the ratio of 30-5-7⁺ to 30-5-7⁻ L^d molecules can be observed by comparing precipitation bands in lanes 1 with 3 for lysates of control cells and lanes 4 with 6 for lysates of cells grown with MCMV peptide.

METHODS. L-L^d cells were incubated with MCMV peptide at 5×10^{-5} M for 15 h at 37 °C, 6.5% CO₂ before labelling. For biosynthetic labelling, L-L^d cells were labelled for 3 h with [³⁵S]methionine²⁸ in the presence of peptide. Cells were lysed with 0.5% Nonidet P-40 and glycoprotein pools were isolated by lentil lectin affinity chromatography. For surface labelling, L-L^d cells were radioiodinated (Na ¹²⁵I) using lactoperoxidase¹⁹. Class I molecules were specifically precipitated using ascites fluid containing the relevant monoclonal antibodies and IgG Sorb (The Enzyme Center). Samples were eluted using 2% SDS and electrophoresed on 10-15% linear polyacrylamide gradient slab gels in the Laemmli buffer system²⁹.

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Identification and characterization of an inhibitor of haemopoietic stem cell proliferation

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THE haemopoietic system has three main compartments: multipotential stem cells, intermediate stage progenitor cells and mature cells. The availability of simple reproducible culture systems¹ has made possible the characterization and purification of regulators of the progenitor cells, including colony-stimulating factors and interleukins. In contrast, our knowledge of the regulators involved in the control of stem cell proliferation is limited. The steady-state quiescent status of the haemopoietic stem cell compartment is thought to be controlled by locally acting regulatory elements present in the stromal microenvironment², but their purification has been hampered by the lack of suitable culture systems. We have recently developed a novel *in vitro* colony assay that detects a primitive cell (CFU-A)³ which has similar proliferative characteristics, in normal and regenerating bone marrow, to the CFU-S (haemopoietic stem cells, as defined by the spleen colony assay⁴) and which responds to CFU-S-specific proliferation regulators. We have now used this assay to purify to homogeneity a macrophage-derived reversible inhibitor of haemopoietic stem cell proliferation (stem cell inhibitor, SCI). Antibody inhibition and sequence data indicate that SCI is identical to a previously described cytokine, macrophage inflammatory protein-1 α (MIP-1 α), and that SCI/MIP-1 α is functionally and antigenically identical to the CFU-S inhibitory activity obtained from primary cultures of normal bone marrow cells⁵. The biological activities of SCI/MIP-1 α suggest that it is a primary negative regulator of stem cell proliferation and that it has important therapeutic applications in protecting haemopoietic stem cells from damage during cytotoxic therapies for cancer.

Preliminary experiments indicated that direct addition of the conditioned medium (CM) from normal bone marrow cells to CFU-A cultures inhibited the formation of macroscopic CFU-A colonies and that SCI activity could be measured on this basis. As earlier studies had identified bone marrow macrophages as possible sources of putative stem cell regulators⁶, we screened murine macrophage cell lines for SCI activity: the CM from one, J774.2 (ref. 7) was particularly effective in inhibiting CFU-A colony formation in this direct addition assay (Table 1). Further analysis revealed that J774.2 CM was also effective in reversibly inhibiting the cycling of stem cells using either *in vitro* (CFU-A) or *in vivo* (CFU-S) assays (Table 1), with regenerating bone marrow as a source of cycling stem cells. J774.2 was therefore selected as a source of SCI for further purification. The CFU-A assay was used to follow the purification of SCI to homogeneity using the methods outlined in the Fig. 1 legend. This procedure resulted in a 50,000-fold purification of the SCI protein from the initial starting material, with yields of ~20%. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the active fractions revealed a protein species that migrated as a doublet with an apparent relative molecular mass (M_r) of 8,000 (8K) (Fig. 1). The fractions that were active in the inhibition of CFU-A colony formation were also shown to significantly reduce the proportion of CFU-A that were

TABLE 1 The effect of unfractionated conditioned media on stem cell proliferation

	% of CFU-A in DNA synthesis	Direct addition to CFU-A (colonies per 10 ⁴ seeded cells)	CFU-Sd12 % in DNA synthesis	CFU-Sd12 per 4 × 10 ⁴ bone marrow cells
Control	37.5 ± 6.4	10.7 ± 2.6	41.3 ± 4.4	10.6 ± 0.8
J774.2	2.0 ± 1.7	2.0 ± 2.9	17.1 ± 5.2	9.9 ± 1.6

CM from J774.2 cell cultures was prepared and concentrated 20-fold over Amicon membranes (M_r cut-off, 10K; the details of this procedure will be described elsewhere (G.J.G. *et al.*, manuscript in preparation). The CFU-A assay was carried out as described previously³. Briefly, normal bone marrow cells in 4 ml α -minimal essential medium containing 25% donor horse serum and 0.3% agar were seeded on top of an underlayer of the same medium containing 0.6% agar, 10% L929 cell CM (a source of colony-stimulating factor-1) and 10% AF1-19T cell CM (a source of granulocyte-macrophage colony-stimulating factor) in a 6 cm Petri dish. Cultures were incubated at 37 °C for 11 days. They were then stained and the numbers of macroscopic CFU-A colonies (composed mainly of macrophages) assessed. CFU-S were assayed as described previously⁴. Spleen colonies were counted on day 12. A suicide technique was used for the assessment of the cycling status of CFU-A and CFU-S³. Regenerating bone marrow cells (sources of cycling stem cells) were incubated in paired tubes, one pair acting as controls and the other pair containing J774.2 CM. The tubes were incubated at 37 °C for 5 h. Cytosine arabinoside was added to one tube from each pair and an equal volume of medium was added to the other for the last 60 min of the incubation. Cells were then washed twice before being assayed for either CFU-A or CFU-S. The numbers of day 12 CFU-S (last column) represent the spleen colony numbers determined after preincubation in the absence of the cytosine arabinoside. All values quoted are means ± s.e.m.

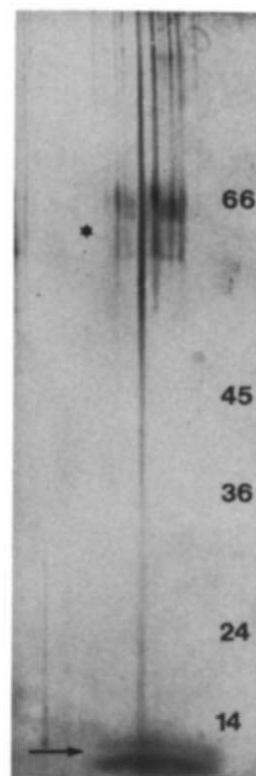


FIG. 1 SDS-PAGE analysis of purified SCI. The position of the SCI doublet is indicated with an arrow and corresponds to the location of SCI activity in SDS-gels as assessed by elution and assay of proteins from gel slices (unpublished observations). Protein bands marked with an asterisk are artefacts resulting from the silver-staining procedure. Migration of M_r markers (in thousands; Amersham Rainbow Markers) is indicated.

METHODS. The details of purification of SCI will be described elsewhere (Graham *et al.*, manuscript in preparation). Briefly, concentrated CM from J774.2 cells was fractionated by ion-exchange chromatography and the active fractions were further resolved through heparin-Sepharose and blue-Sepharose affinity columns. SCI purified by this method was visualized on SDS-polyacrylamide gels (15%)²⁵ and stained using the silver-staining method described previously²⁶.

|| To whom correspondence should be addressed.

TABLE 2 Biological activity of SCI and MIP

Cytokine	% CFU-A in DNA synthesis	Direct addition	
		CFU-A assay (colonies per 10^4 bone marrow cells)	GM-CFC assay (Colonies per 2.5×10^4 bone marrow cells)
SCI			
Control	35.6 ± 4.8	9.26 ± 3.3	43.2 ± 5.7
SCI	$19.6 \pm 9.95^*$	2.5 ± 0.9	50.4 ± 11.4
MIP			
Control	39.2 ± 2.9	11.0 ± 1.9	47.8 ± 13.0
MIP-1	$20.2 \pm 5.3^*$	0.5 ± 0.5	not tested
rMIP-1 α	$18.8 \pm 2.3^\dagger$	0.8 ± 0.8	42.8 ± 11.1
rMIP-1 β	43.8 ± 4.4	12.8 ± 3.3	44.3 ± 11.7

All values quoted are means $\pm$ s.e.m. * Significant $P < 0.02$; † significant $P < 0.01$; two-tailed t -test. SCI was purified as described elsewhere (G.J.G. *et al.*, manuscript in preparation). For the MIP tests, MIP-1 α and MIP-1 β were CM from COS cells previously transfected with the cloned cDNAs. MIP-1 was used as purified protein⁸. All these preparations were used at optimal concentrations in the direct addition assays and at suboptimal concentrations in the cycling status assays. Controls were either CM from mock-transfected COS cells or medium. The CFU-A direct addition assay and the determination of the cell cycle status of CFU-A were performed as described in the Table 1 legend. Regenerating bone marrow was used as a source of cycling stem cells in the cell cycle status assay. GM-CFC assays were performed as previously described³ using recombinant murine GM-CSF as a stimulant.

TABLE 3 The effect of SCI on CFU-S proliferation

Group	%CFU-S in DNA synthesis	CFU-S per 10^5 bone marrow cells
Control*	44.2 ± 4.7	22.6 ± 0.6
Inhibitor †	$9.4 \pm 8.2^\P$	21.4 ± 0.8
Inhibitor † + anti-MIP-1 $\ddagger$	51.0 ± 17.4	20.0 ± 1.8
rMIP-1 α	$7.1 \pm 7.1^\P$	21.2 ± 2.8
rMIP-1 α + anti MIP-1 $\ddagger$	41.8 ± 6.1	20.8 ± 2.4
Anti MIP-1 $\ddagger$	45.4 ± 10.4	22.0 ± 1.3

CFU-S assays and cell cycle status measurements were carried out as described in the Table 1 legend. MIP-1 antibody was prepared as previously described for MIP-2²⁷. The antibody was a protein-A-purified IgG fraction of a rabbit polyclonal antibody produced against purified MIP-1 protein. When it was added, this antibody was present throughout the incubation period.

* Regenerating bone marrow³ used as a source of cycling stem cells.

† $50 \mu\text{g ml}^{-1}$ NBME-IV a source of normal bone marrow-derived SCI⁵.

¶ $20 \mu\text{l ml}^{-1}$ equivalent to 1:50 dilution.

|| $400 \mu\text{l ml}^{-1}$ of the COS CM (see Table 2). Results are mean $\pm$ s.e.m. for two experiments. * $P < 0.01$; two-tailed t -test.

cycling (Table 2, top). Finally, the proliferation of more mature lineage-restricted progenitors such as granulocyte-macrophage colony-forming cells (GM-CFC) was shown to be unaffected (Table 2, top), demonstrating the stem cell specificity of the inhibition. The biological activities found in J774.2 CM and normal bone marrow extracts were therefore observed in the most highly purified fractions. The purified SCI doublet was resolved into its two component peptides using reverse-phase high-pressure liquid chromatography and both were analysed by N-terminal sequencing. A major sequence of 20 residues (APYGADTPTAXXFSYSRKIP; one-letter amino-acid code) representing the more abundant, lower- M_r peptide and a minor sequence of nine residues {APMXS (D/P)P(P/T)} derived from the peptide of higher M_r were obtained. Similarities in the M_r and biochemical properties of SCI and MIP-1, which had been recently described⁸, led us to compare these N-terminal sequences with those for MIP-1 α ⁹ and MIP-1 β ¹⁰, the component peptides of the MIP-1 doublet. The major sequence from our

SCI preparation corresponded to MIP-1 α and the minor one to that of MIP-1 β . Complementary DNAs from J774.2, encoding MIP-1 α and MIP-1 β , were amplified by the polymerase chain reaction¹¹, using 25-nucleotide oligomer primers beginning at the initiation codon and starting from the antisense sequence of the stop codon. These primers therefore contained the full coding sequences of both peptides. The oligonucleotide sequences were derived from previously published sequences^{9,10}. Restriction site sequences in the oligonucleotides allowed ligation of the reaction products into a COS cell expression vector, pXM (ref. 12). A single clone from each reaction was sequenced and the identity of these clones with sequences published for either MIP-1 α or MIP-1 β was confirmed. The cDNA constructs were then transfected into COS cells and the biological activity of the recombinant material tested (Table 2, bottom). The results showed that purified native MIP-1 and recombinant MIP-1 α (rMIP-1 α), but not rMIP-1 β , reversibly decrease the cycling status of CFU-A (Table 2, bottom). Neither rMIP-1 α nor rMIP-1 β had any detectable effect on GM-CFC progenitors (Table 2, bottom). The rMIP-1 α was also tested in the *in vivo* CFU-S assay (Table 3) and the results confirm those obtained with the *in vitro* CFU-A assay. Furthermore, a neutralizing serum prepared against native MIP-1 blocked both rMIP-1 α activity and the SCI activity from normal bone marrow cell CM⁵ (Table 3). The inhibitor preparation does not seem to have a toxic effect on CFU-S, given that CFU-S numbers are not affected by preincubation with the inhibitor (Tables 1 and 3). We concluded that MIP-1 α and J774.2-derived SCI are identical and represent a CFU-S-specific proliferation regulator which is functionally and antigenically identical to the SCI released by normal bone marrow cells⁴. It is interesting that the native M_r of SCI released by J774.2 cells is in the range 50–100K (as measured by gel filtration), which is similar to the M_r of the *in vivo*-derived material. It is therefore likely that SCI exists as a high- M_r complex under physiological conditions.

The cytokine we have characterized would have the ability to reduce the proportion of haemopoietic stem cells in DNA synthesis specifically, thereby protecting them from cytosine arabinoside, a cell-cycle-specific cytotoxic drug. In contrast, SCI does not affect the proliferation of more mature progenitors and so appears to be a specific regulator of the stem cell compartment as defined by the day 12 CFU-S. There have been reports of a tetrapeptide that inhibits haemopoietic stem cell proliferation by preventing recruitment of CFU-S into the cell cycle^{13,14}. Although this low- M_r peptide might arise *in vivo* through proteolytic maturation of a 'precursor' protein¹⁴, the SCI reported here is clearly not related to the tetrapeptide in either structure or mechanism of action. Lord *et al.* have postulated that the regulation of CFU-S proliferation is mediated by an inhibitor⁵ produced by normal bone marrow cells that maintains the normal quiescent status, and a stimulator detected in regenerating bone marrow that switches stem cells into the cell cycle¹⁵. Furthermore, it has been suggested that the CFU-S proliferative status may be modulated by the relative levels of inhibitor and stimulator². Our data identify a CFU-S-specific proliferation regulator, and it should therefore now be possible to test this hypothesis more rigorously with SCI molecular probes.

Studies of the primary structure of the MIP polypeptides have shown that they are members of a large family of cytokines^{8,9,10,16}. The biological activities of most members of this family have yet to be elucidated although some, such as MIP-1, have been tentatively designated inflammatory mediators. Although the inflammatory response to MIP-1 apparently requires micromolar concentrations^{8,16}, our data (unpublished observations) indicate that SCI is active in the 200–500pM range, as measured in the CFU-A direct addition assays. SCI displays a linear dose-response relationship in this assay, with a plateau of activity at nanomolar concentrations. The unexpected discovery that rMIP-1 β , which has 67% amino-acid sequence identity with MIP-1 α /SCI, has no detectable

effect on stem cell proliferation (Table 2, bottom) at similar concentrations, does emphasize the specificity of SCI and merits further investigation. It is possible that the SCI activity described here for the MIP-1 α protein will provide clues to the physiological roles of the other members of this family¹⁶ whose genes have been assigned to mouse chromosome 11, where the genes for other haemopoietic regulators, including GM-CSF and interleukin-3, are located (S. Wilson and M. Dorf, personal communication).

A stem cell inhibitory activity, similar if not identical to murine SCI, has been reported to be released by normal human bone marrow cells¹⁷. It is therefore probable that the murine studies

described here will have direct relevance to the control of human haemopoietic stem cell proliferation. An important implication of our results is the potential for SCI to protect stem cells *in vivo*¹⁸ against the effects of cytotoxic drugs used for cancer therapy. Furthermore, as the effectiveness of chemotherapy correlates with dose intensity and treatment is dose-limited by myelotoxic effects^{19,20}, administration of SCI might allow more intensive therapy. Experiments with recombinant SCI in pre-clinical trials will allow this possibility to be tested. The availability of various stem cell purification procedures²¹⁻²⁴ and of recombinant SCI will now allow a more precise evaluation of the mode of action of this inhibitor and its target cells. □

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Expression of an erythroid transcription factor in megakaryocytic and mast cell lineages

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THE nuclear factor GF-1 (also known as NF-E1, Eryf-1; refs 1-3 respectively) is important in regulation of the transcription of globin and other genes⁴ that are specifically expressed in erythroid cells. We have previously shown that GF-1 of both mouse⁵ and human⁶ origin is a 413-amino-acid polypeptide with two novel zinc-finger domains whose expression is restricted to erythroid cells^{5,6}. Using *in situ* hybridization of mouse bone marrow cells and northern blot analysis of purified cell populations and permanent cell lines, we show here that GF-1 is expressed in two other haematopoietic lineages, megakaryocytes and bone marrow-derived mast cells. Our findings are consistent with results from haematopoietic progenitor culture which suggest a relationship between erythroid, megakaryocytic and mast cell lineages, and imply that GF-1 is expressed in committed multipotential cells and their progeny. Hence, the mere presence of this transcription factor is unlikely to be sufficient to programme differentiation of a single haematopoietic lineage. GF-1 may regulate the transcription of not only erythroid genes, but also many genes characteristic of megakaryocytes and mast cells, or genes shared among these lineages.

GF-1 messenger RNA transcripts and DNA-binding activity are present in mouse and human erythroleukaemia cell lines^{1,2,5,6}, as well as in avian erythrocytes at varying developmental stages⁷, but are not found in myeloid, monocytic, lymphoid or non-haematopoietic cell lines of either mouse or human origin^{5,6}. To assess the pattern of GF-1 expression in normal cells, rather than in transformed cell lines or mixed cellular tissues, we hybridized⁸ mouse bone marrow cells *in situ* with ³⁵S-labelled RNA synthesized from cloned complementary DNA. Cultured mouse erythroleukaemia (MEL) cells served as a positive control cell. Nonspecific background was evaluated by hybridization with ³⁵S-labelled sense RNA. As shown in Fig. 1a and b, GF-1 RNA was detected in MEL cells by hybridization with the antisense but not with the sense probe. In mouse bone marrow, we identified cells under brightfield optics that could be classified as late erythroid (normoblasts), indeterminate early precursors ('blasts'), megakaryocytes ('megs') or neutrophilic granulocytes ('polys'), as described in the legend to Fig. 1. Hybridization was observed with megakaryocytes and blasts, and was weaker with normoblasts. Examples are shown in Fig. 1c and d, with negative controls in e and f. To quantitate this observation, we counted grains over the cytoplasm and within 2.5 μ m of the cell membrane as cell-associated, without correcting for cell size. Data from two independent experiments (Fig. 2a, b) confirmed our assessment of megakaryocytes, normoblasts and blasts. The strong signal from megakaryocytes compared with MEL or normoblasts might reflect their large size, rather than an increased abundance of GF-1 RNA relative to erythroid cells. As their morphology deteriorated somewhat during the *in situ* hybridization procedure, early erythroid and myeloid precursor forms, as well as some lymphoid cells, could not be distinguished in the blast population. We suspect that GF-1 RNA expression in early erythroid blasts accounts largely (if not exclusively) for the signal in the blast group. Considering this with its absence in permanent myeloid and lymphoid mouse or human cell lines, we conclude that among differentiated marrow cells, GF-1 expression is restricted to erythroid cells and megakaryocytes.

GF-1 expression in megakaryocytes was verified by northern blot analysis of RNA from purified platelets and megakaryocytic human leukaemic cell lines⁹. As shown in Fig. 3a, rat platelets

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contain GF-1 mRNA indistinguishable in size from that in MEL cells. We have also detected DNA-binding activity identical to GF-1 in extracts of purified rat megakaryocytes (data not shown). Human leukaemic cell lines with erythroid-megakaryocytic (K562 and HEL) or only megakaryocytic (CHRF-288; ref. 10) characteristics also contain GF-1 mRNA (Fig. 3*b*) and GF-1 gel shift activity (Fig. 3*c*). Furthermore, our conclusion that GF-1 is expressed in the megakaryocytic lineage is supported by independent data of Romeo *et al.*¹¹.

Bone marrow cell progenitor assays have indicated that random commitment to specific lineages is sequential during differentiation from a pluripotent haematopoietic stem cell¹². Also, there is a close relationship of the erythroid, megakaryocytic and mast cell lineages, and the existence of a multipotential committed progenitor (designated EMmast) can be inferred^{12,13}. As we could not identify mast cells in the *in situ* hybridization of marrow, we analysed RNA from purified, bone-marrow derived mouse mast cells by northern blotting (Fig. 3*d*);

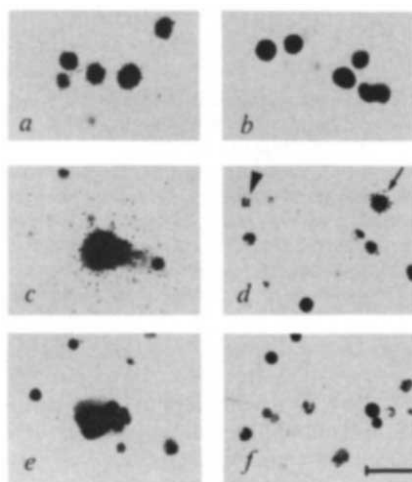


FIG. 1 *In situ* detection of GF-1 mRNA in murine haematopoietic cells. Autoradiographs of histological sections are shown. MEL (*a, b*) cells or murine bone marrow suspensions (*c-f*) were embedded in agarose, sectioned, and hybridized to ³⁵S-labelled antisense (*a, c, d*) or an equivalent concentration of negative control sense (*b, e, f*) orientation GF-1 RNA probes. The signal appears as discrete silver grains associated with specific cell types. Cell types identified in our analysis included: segmented neutrophilic granulocytes (polys); normoblasts, late stage erythroid cells with round, pyknotic nuclei (normo); indeterminate precursors with large pachychromatic, slightly irregular or folded nuclei and a moderate amount of cytoplasm (blasts); and megakaryocytes (megs). Eosinophils were not identified or scored in this analysis. GF-1 message is readily detected in megakaryocytes (*c*) and blasts (*d*, arrowed), but is not visible in most other cell types, including neutrophils (*d*, denoted by arrowhead). Panels *a, c* and *d* are from a single histological section, as are *b, e* and *f*. Exposure time, 26 days. Scale bar is 20 μ m.

METHODS. For *in situ* hybridization, cultured MEL cells or murine bone marrow cells (obtained by flushing the marrow cavity) were washed and embedded in agarose before fixation as follows. A 1.5-mm thick underlayer of 1% agarose was prepared in a Petri dish, allowed to harden, and a second equally thick layer poured. Holes 5–8 mm in diameter were punched in the upper layer to form a well with a flat base. A slurry was made from cell pellets and micropipetted into an equal volume of 1.2% low-melting-point agarose made in phosphate-buffered saline with 1% Dextran blue 2000 at 37 °C. MEL cells and bone marrow cells were micropipetted into separate agarose wells within the same Petri dish. When the suspension had hardened, it was overlaid with standard-melting-point agarose, cut into a rectangular block, sealed by searing the edges with a hot spatula, and immediately fixed. The protocol for hybridization of ³⁵S-labelled RNA probes to cellular RNAs in 6–8 μ m-thick paraffin tissue sections has been described⁸. For synthesis of radioactive RNA probes, the entire *Xho*I insert of pXM GF-1 clone 127 (ref. 5) was subcloned into the *Sal*I site of Bluescript (Stratagene). Antisense probe was prepared by T3 polymerase and sense probe by T7 polymerase transcription²¹ after linearization of plasmid with *Bam*HI or *Xho*I, respectively.

these also express GF-1 RNA. Thus, expression of this factor is seen in three differentiated cell types, which are probably descended from a common, committed progenitor.

Several findings demonstrate that the GF-1 RNA in megakaryocytic and mast cell lineages is the same as that found in erythroid cells. As only a single X-linked GF-1 gene is detected in mouse or human genomic DNAs by high-stringency hybridization⁶, the transcripts seen in megakaryocytic or mast cells (Fig. 3*a, b, d*) cannot be products of a different gene. In addition, S1 nuclease mapping and RNase protection assays of mouse erythroid and mast cell RNAs, which includes all intron-exon boundaries and examination of promoters (S.-F. Tsai and S.H.O., unpublished results), demonstrate no structural differences (data not shown). Furthermore, by combining cDNA cloning and S1 nuclease mapping, we find that the predominant GF-1 RNA in both human erythroleukaemic and megakaryocytic cells corresponds in structure to the mRNA first characterized in MEL cells. Specifically, cDNA clones derived from a library prepared from phorbol ester (TPA; 12-*O*-tetradecanoylphorbol-13-acetate)-treated (megakaryocytic) HEL cells are identical to clones from a K562 cell library and also colinear with MEL clones. In the course of this analysis, however, we encountered variant cDNA clones, present in both human cDNA libraries, that precisely lacked sequences derived from exon-2 of the gene (L.Z., S.-F. Tsai and S.H.O., unpublished data). As shown in Fig. 4, both the predominant and variant RNAs are found in erythroleukaemic and megakaryocytic-only cell lines. The significance of this alternative splicing of GF-1 RNA in human cells is still uncertain, but the salient observation is that the same erythroid, full-length form of GF-1 RNA is expressed in both mouse erythroid and mast cells and in human erythroleukaemic and megakaryocytic cell lines. Thus, a single GF-1 gene is transcribed in three haematopoietic lineages into a single predominant RNA.

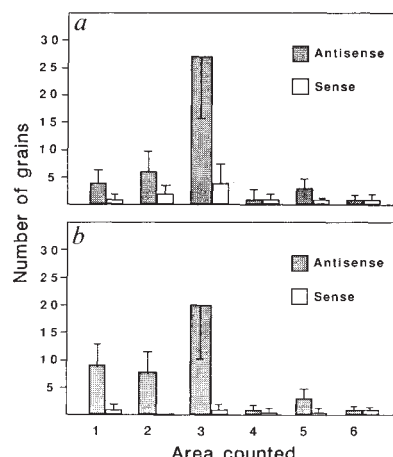


FIG. 2 Quantitation of the *in situ* hybridization signal of GF-1 to bone marrow cell types. Autoradiograph grains associated with specific cell types were counted in two independent experiments (*a, b*). Each bar pattern (1–6) represents a single slide. MEL cells were included as a positive tissue control. As a negative control, an identical quantity of sense GF-1 probe was hybridized to sections from each block. Emulsion background was evaluated by counting grains within a 25 μ m square ocular grid in areas with no cells (6). This analysis confirmed the visual impression that MEL cells (1), blasts (2) and megakaryocytes (3) exhibit a positive GF-1 signal. In addition, grain counting permitted analysis of cell types associated with fewer grains. In particular, neutrophils (4) had no significant signal above background sense probe negative controls. Lastly, erythroid cells (5) hybridize to GF-1 probe with a signal (antisense probe) to noise (sense probe) grain ratio of 2.9 in experiment *a* and 13.3 in experiment *b*. Autoradiographic silver grains in slides developed after 35 (experiment *a*) or 26 (*b*) days exposure were counted at $\times 400$ magnification with darkfield optics. Standard deviations are indicated either above or below the bars.

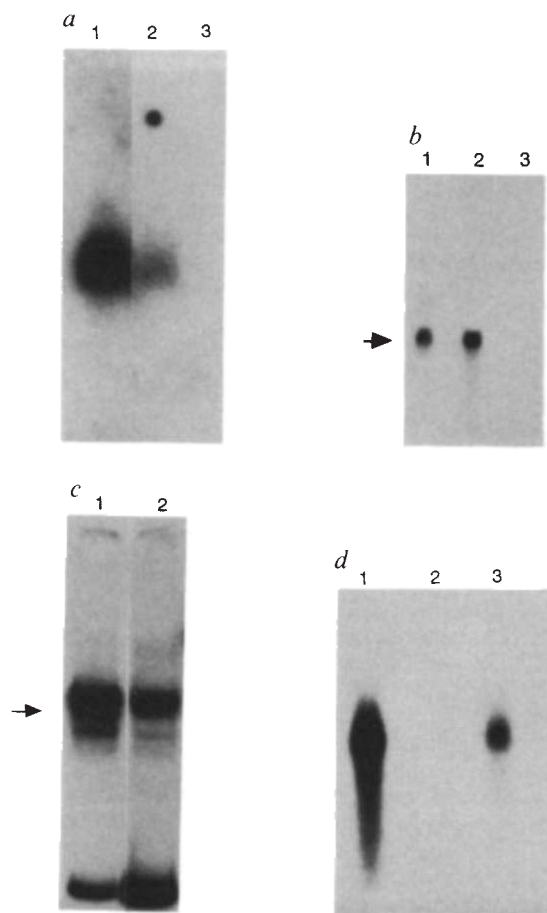


FIG. 3 Expression of erythroid factor RNA and protein in platelets, human leukaemic cell lines, and mast cells. *a*, Northern blot analysis of RNA from MEL cells (lane 1), rat platelets (lane 2) and 3T3 mouse fibroblasts (lane 3). Total RNA (10 μ g) was subjected to northern blot analysis²¹ using mouse cDNA as probe. *b*, Northern blot analysis of RNAs from haemin-treated human erythroleukaemia K562 (lane 1), megakaryocytic-only CHRF-288 (lane 2) and Hela (lane 3) cells. Human GF-1 cDNA was used as probe. GF-1 RNA is indicated by the solid arrow. The minor species of slower mobility in lane 1 is variably seen in erythroleukaemic cells treated with either haemin or TPA⁶. *c*, Detection of GF-1 DNA-binding activity in megakaryocytic-only CHRF-288 cells. Nuclear extracts of K562 (lane 1) and CHRF-288 (lane 2) cells were incubated with a 117-base pair labelled *Hinf*I/*Nco*I fragment of the human A γ -globin gene and electrophoresed, as previously described¹, to reveal sequence specific DNA-binding characteristic of GF-1. The major DNA-GF-1 complex is indicated by the arrow. The minor complex below the main band is thought to result from binding of a proteolytic fragment of GF-1 (ref. 5). *d*, Northern blot analysis of RNAs from MEL cells (lane 1), mouse fibroblasts (lane 2), and purified mouse (C57b1/6) mast cells (lane 3). Mast cells were purified from bone marrow by A. Reith as described²².

Recently the restriction of some cell-specific transcription factors to a single cell lineage has come into question. For example, Oct-2 expression, previously thought to be limited to lymphoid cells, has been observed in embryonic brain¹⁴ and in adult brain and kidney¹⁵. As we have not detected GF-1 expression in non-haematopoietic cell types, its presence in three haematopoietic lineages of presumably common origin, but not in myeloid or lymphoid cells, has several implications for its role in gene expression and differentiation.

First, GF-1 may be active in the committed EMmast progenitor whose existence is indicated by bone marrow progenitor culture studies¹². All of the differentiated cell types that tested positive, which are probably descended from a common committed cell, express the factor. In addition, permanent cell lines that express the factor may have characteristics of erythroid cells and megakaryocytes (K562, HEL, OCI (ref. 16), Mo-7 (ref.

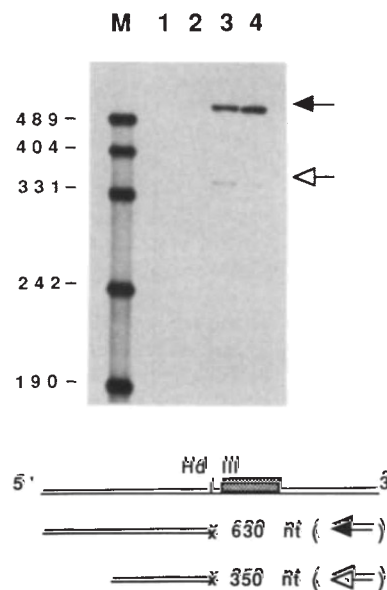


FIG. 4 Detection of full-length and alternatively processed human GF-1 RNAs. Lanes: M, DNA markers (sizes in kb on the left); 1, tRNA; 2, from Hela; 3, from K562; 4, from CHRF-288. RNA (20 μ g) was mapped using S1 nuclease²¹ and a 5' end-labelled DNA probe spanning the 5' half of the GF-1 cDNA. Samples were digested with 500 U ml⁻¹ S1 nuclease after hybridization and electrophoresed through an 8% urea-acrylamide gel. The diagram depicts protected fragments derived from hybridization to the full-length (closed arrow) and alternatively processed (open arrow) RNA species. The DNA-binding domain is indicated by the hatched box. nt, Nucleotides.

17) DAMI (ref. 18) or erythroid and mast cells (32D and B6SutA; ref. 19, and unpublished data). We speculate that GF-1 might mediate commitment to these lineages, and that it is necessary for their continued differentiation. It is less plausible that expression of this factor should be activated after commitment to the individual lineages.

Second, expression of GF-1 alone cannot be sufficient to specify differentiation of any of the lineages in which it is expressed. In its simplest terms, this conclusion contrasts with the proposed role of a DNA-binding protein, such as MyoD (ref. 20), which may programme lineage-specific differentiation as a single agent when expressed in the appropriate environment. We infer from our studies that GF-1 probably acts with other cell-restricted transcription factors to direct cell-type specific expression in the haematopoietic lineages in which it is found. Such combinations, perhaps in association with changing levels of GF-1 expression, may provide the requisite transcriptional specificity.

Third, by analogy to the involvement of GF-1 in globin gene expression in erythroid cells, we speculate that this factor may participate in transcription of a variety of genes expressed specifically in megakaryocytes or mast cells, or shared between two or more of the lineages. One candidate for a gene whose expression is shared between lineages is the erythropoietin receptor gene, as it is expressed in both erythroid cells and megakaryocytes, and also contains a consensus binding site for GF-1 in its proximal promoter (Y. Youssoufian, L.I.Z., S.H.O., A. D. Andrea and H. Lodish, manuscript submitted). The presence of GF-1 binding sites in the promoters of genes specifically expressed in megakaryocytes, as described by Romeo *et al.*¹¹, provides further support for this concept.

Overall, our findings indicate that GF-1 is a transcriptional regulator for a subset of haematopoietic lineages, rather than solely an erythroid factor. This conclusion suggests that the protein has a broader role in haematopoietic cell development than was previously envisioned. □

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Megakaryocytic and erythrocytic lineages share specific transcription factors

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ERYTHROID-specific genes contain binding sites for NF-E1 (also called GF-1 and Eryf-1; refs 1–3 respectively), the principal DNA-binding protein of the erythrocytic lineage. NF-E1 expression seems to be restricted to the erythrocytic lineage⁴. A closely related (if not identical) protein is found in both a human megakaryocytic cell line and purified human megakaryocytes; it binds to promoter regions of two megakaryocytic-specific genes. The binding sites and partial proteolysis profile of this protein are indistinguishable from those of the erythroid protein; also, NF-E1 messenger RNA is the same size in both the megakaryocytic and erythroid cell lines. Furthermore, point mutations that abolish binding of NF-E1 result in a 70% decrease in the transcriptional activity of a megakaryocytic-specific promoter. We also find that NF-E2, another *trans*-acting factor of the erythrocytic lineage, is present in megakaryocytes. Transcriptional effects in both lineages might then be mediated in part by the same specific *trans*-acting factors. Our data strengthen the idea of a close association between the erythrocytic and the megakaryocytic lineages and could also explain the expression of markers specific to the erythrocytic and megakaryocytic lineages in most erythroblastic and megakaryoblastic permanent cell lines^{5,7}.

Only three well characterized genes are highly specific of megakaryocytopoiesis: those coding for human platelet glycoproteins IIb (GPIIb; ref. 8), Ib α (GPIb α ; ref. 9) and rat platelet factor 4 (PF4; ref. 10). Human platelet glycoprotein IIb is the α -subunit of the platelet Arg-Gly-Asp-sensitive integrin (GPIIb-IIIa) which functions as a receptor for fibrinogen, fibronectin and von Willebrand factor at the surface of plate-

lets¹¹. The human GPIIb gene is expressed both in a megakaryocytic-specific cell line (MEG 01; ref. 12) and in an erythrocytic/megakaryocytic cell line, HEL^{13,14}. A 330-base pair (bp) DNA fragment spanning nucleotides from –305 to +26 relative to the transcriptional start site of the human GPIIb gene was made by polymerase chain reaction, starting from human DNA and using two oligonucleotides complementary to the –305 and +26 sequences. After electroelution, the DNA fragment was used as a template for a second polymerase chain reaction, with one unlabelled and one labelled oligonucleotide. This gave a probe of very high activity and, as there was no cloning step, the *Taq* polymerase errors were reduced to background. The ability of this fragment to bind nuclear factors was assessed by DNase I footprint assay using crude nuclear extracts from HeLa, K562 or MEG 01 cell lines. These lines were chosen because HeLa cells do not express either an erythroid or a megakaryocytic-specific marker, K562 cells express only erythroid markers and not megakaryocytic-specific markers (these cells are negative for GPIIb and GPIb α ; data not shown), whereas MEG 01 cells express megakaryocytic-specific markers (GPIIb, GPIb α) but no erythroid-specific marker (<1% of the cells are glycophorin A-positive, and we found no glycophorin A mRNA in this cell line; data not shown). Regions protected from DNase I digestion are shown in Fig. 1. A first footprint, around nucleotide –15, was present in all extracts and covered a purine-rich region of the GPIIb promoter. A second footprint (in the nucleotide –40 region) was weak but was found only with K562 or

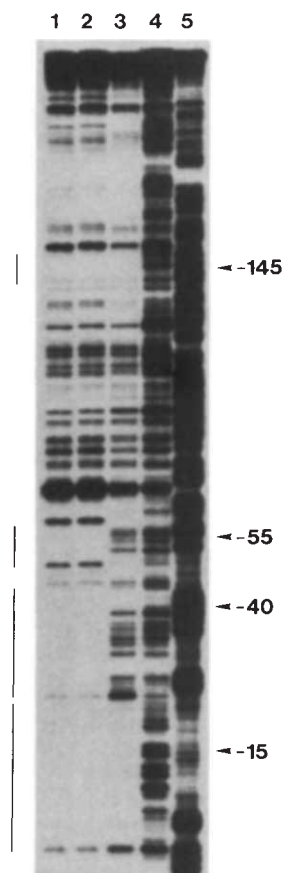


FIG. 1 Analysis of protein-DNA interactions at the human GPIIb promoter by DNase I footprinting. The –305/+26 probe was labelled on the coding strand at position +26. It was incubated with 10 μ g crude nuclear extract from either MEG 01 (lane 1), K562 (lane 2) or HeLa (lane 3) cell lines followed by footprinting as described¹⁶. Lane 4 is a DNase I digestion of the probe without extract. Lane 5 is a (G+A) chemical degradation sequencing ladder of the probe. Locations of protein-binding sites are shown on the left.

MEG 01 extracts. This footprint covered a region containing the motif 5'-TTCCT(N)₄AGGAA-3', a perfect inverted repeat. A third footprint (in the -55 region), surrounded by two strong hypersensitive sites, was present in extracts from K562 and MEG 01 but not from HeLa cells. It is centred on the sequence 5'-TGATAA-3' located at -55 in the GPIIb promoter. This sequence is the core sequence recognized by NF-E1, the main DNA-binding protein of the erythroid lineage. Finally, a fourth footprint (in the -145 region) was evident for all cell extracts and lay over a perfect CAC consensus sequence. The CAC-NF-E1 association is found in many erythroid-specific promoters and might be relevant for megakaryocytic-specific promoters, although the distance between these two elements is much larger in the GPIIb promoter (85 bases) than it is in the erythroid-specific promoters previously described¹⁵ (between 12 and 30 bases).

We characterized by gel retardation the protein that binds the NF-E1 consensus sequence of the GPIIb promoter and looked for a similar binding reaction in another megakaryocytic-specific promoter, the promoter for rat platelet factor 4 (Fig. 2a). A 30-bp oligonucleotide centred on the NF-E1 core sequence of the human GPIIb promoter and a 30-bp oligonucleotide centred on a putative NF-E1 binding site located 30 bp upstream from the initiation site of the rat platelet factor-4 gene were incubated with the different cell extracts and electrophoresed. We observed a strong mobility shift after incubation of those oligonucleotides with nuclear extracts, which was identical for K562 and MEG 01 (Fig. 2a, lanes 1, 6 and 2, 7) but different from HeLa cells. A human porphobilinogen deaminase (PBGD) NF-E1-binding oligonucleotide¹⁶ (Fig. 2a, lanes 3 and 8) and the GPIIb and PF4 oligonucleotides (Fig. 2a, lanes 4, 9 and 9, 10) could all compete with it. NF-E1 expression in megakaryocytic cell lines was also demonstrated by partial proteolysis and northern blot analysis of RNA isolated from MEG 01 and K562: the pattern of proteinase K digestion is identical for NF-E1 from both (data not shown), so are the sizes of their NF-E1 mRNAs (Fig. 2b). We conclude that

both GPIIb and platelet factor-4 promoters have strong NF-E1-binding sites and that megakaryocytic cell lines express NF-E1.

Either the NF-E1 binding found in MEG 01 was an effect of cell transformation or it was a genuine megakaryocyte activity. We assayed a nuclear extract from purified human megakaryocytes for NF-E1 binding activity. As shown in Fig. 3, the activity is comparable in megakaryocytes and in MEG 01 cells, indicating that NF-E1 is functional during normal megakaryopoiesis. The reported presence of NF-E1 mRNA in megakaryocytes is consistent with our results¹⁷.

As NF-E1 from both erythrocytic and megakaryocytic lineages will bind to DNA, we next asked whether the *cis*-acting sequence recognized by NF-E1 is involved in transcription from the human GPIIb promoter. Using site-directed mutagenesis on a -112/+33 bp DNA fragment of the human GPIIb promoter, we generated an NF-E1⁻ mutant of the GPIIb promoter by two transversions in the -55 TGATAA sequence, thereby creating a -55 TGCGAA motif not recognized by NF-E1. The mutant and the wild-type promoters were then linked to the chloramphenicol acetyltransferase (CAT) reporter gene and transfected into HEL cells, together with a plasmid containing the firefly luciferase gene under the control of the Rous sarcoma virus promoter. Normalization of CAT activity shows that the NF-E1⁻ promoter reproducibly decreases transcription by 70%; so NF-E1 is important for activation of megakaryocyte as well as erythroid-specific promoters.

To investigate whether there are other *trans*-acting factors in the erythrocytic and megakaryocytic lineages apart from NF-E1, we looked for NF-E2 in MEG 01 cells and in purified megakaryocytes. NF-E2 binds to the erythroid-specific promoter of the PBGD gene¹⁶ and to the dominant control region upstream of the embryonic human ϵ -globin gene. It recognizes an AP1 consensus sequence; we have already shown that NF-E2 is not a proteolytic fragment of AP1 (ref. 15). Indeed, we find an NF-E2 activity in these megakaryocytic cells (Fig. 4), indicating that the erythroid and the megakaryocytic lineages probably

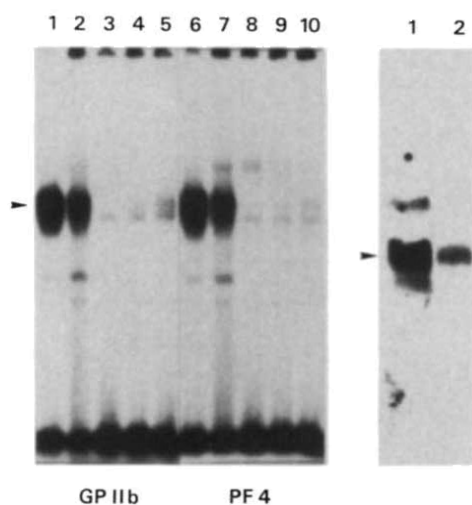


FIG. 2 a, Two megakaryocytic-specific genes contain NF-E1-binding sites. ³²P-labelled oligonucleotides that contain either the -69 to -39 sequence of the human GPIIb promoter (lanes 1 to 5) or the -44 to -14 sequence of the rat platelet factor-4 promoter (lanes 6 to 10) were incubated with nuclear extract (12 μ g) from K562 cells (lanes 1 and 6) and MEG 01 cells (lanes 2 and 7). MEG 01 nuclear extract and 50 ng of the -70 NF-E1 binding site of the human erythroid PBGD promoter (lanes 3 and 8), the GPIIb oligonucleotide (lanes 4 and 9) and the PF4 oligonucleotide (lanes 5 and 10) were used in competition experiments. b, Northern blot analysis of human erythroid or megakaryocytic cell lines. Total RNA (20 μ g) was analysed by northern blotting¹⁸ using human NF-E1 cDNA as probe. Lane 1, K562; lane 2, MEG 01. The band corresponding to NF-E1 mRNA is shown.

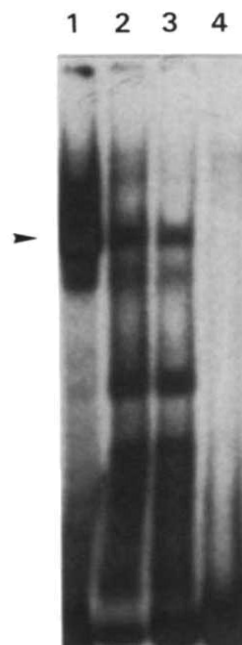


FIG. 3 Purified megakaryocytes contain an NF-E1 binding activity. A ³²P-labelled oligonucleotide that contains the -60 to -39 sequence of the human GPIIb promoter was incubated with nuclear extracts from MEG 01 cells (lane 1) or from megakaryocytes purified as described¹⁹ (lanes 2 (10 μ g) and 3 (20 μ g)). Competition for the 70 NF-E1 binding site of the human erythroid PBGD promoter (50 ng) was carried out using a megakaryocyte nuclear extract (lane 4). The arrow indicates the NF-E1 complex.

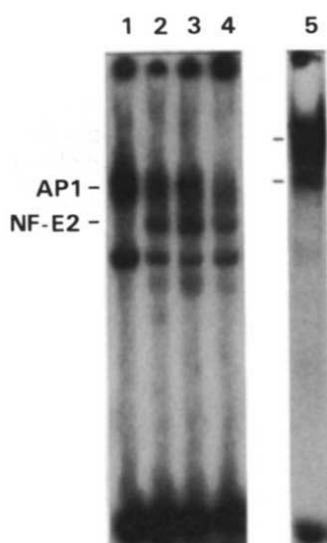


FIG. 4 Megakaryocytic cell lines and megakaryocytes also contain an NF-E2 binding activity. The -160 AP1/NF-E2 binding site of the human PBGD erythroid promoter was labelled, incubated with crude nuclear extract from HeLa cells (20 μ g; lane 1), K562 cells (12 μ g; lane 2), MEG O1 (12 μ g; lane 3), HEL cells (12 μ g; lane 4) or purified megakaryocytes (12 μ g; lane 5) and loaded on 4% (lanes 1, 2, 3 and 4) or 6% (lane 5) polyacrylamide gels. The bands corresponding to the AP1 and NF-E2 complexes are shown. The band immediately below corresponds to a non-specific complex.

share many *trans*-acting factors not found in the other haematopoietic lineages.

There are two conclusions to be drawn from our results. First, either NF-E1 is present in a committed erythrocytic-megakaryocytic precursor, or it is activated in a similar way in the erythrocytic and megakaryocytic lineages. Second, despite the presence of *trans*-acting factors involved in the regulation of erythroid-specific genes, no globin, glycophorin A or erythroid PBGD mRNA is found in megakaryocytes. This could be explained by the presence in megakaryocytes of a still unknown repressor of erythroid-specific genes; alternatively, erythroid cells could possess a specific pathway of *trans*-activation not found in megakaryocytes. Clarification of these points will be crucial to our understanding of the molecular mechanisms in haematopoietic differentiation. □

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Direct measurement of exocytosis and calcium currents in single vertebrate nerve terminals

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THE release of neurohormone is widely thought to be exocytotic, involving Ca^{2+} -dependent^{1,2} fusion of secretory vesicles^{3,4} with the plasma membrane⁵. The inaccessibility of most nerve endings has so far hampered direct time-resolved measurements of neuronal exocytosis in response to brief depolarization. By using 'whole-terminal' patch-clamp and circuit-analysis techniques to measure membrane capacitance⁶, we have now monitored changes in the surface membrane area of individual nerve terminals isolated from the mammalian neurohypophysis⁷. A single depolarizing pulse leading to Ca^{2+} entry through voltage-gated calcium channels⁸, rapidly and reproducibly increases the membrane area by an amount corresponding to the fusion of 1-100 secretory vesicles. The magnitude of the capacitance increase depends not only on Ca^{2+} entry and buffering, but also on the pattern of stimulation revealing facilitation, fatigue and recovery of the release process.

The neurohypophysis contains the endings of hypothalamic neurons which release the peptides oxytocin and vasopressin in response to bursting patterns of electrical activity⁹. These endings have been isolated¹⁰, yielding a preparation of spherical terminals (1-12 μ m in diameter)¹¹ that release these peptides in a calcium-dependent manner in response to depolarization¹⁰. We have studied Ca^{2+} -triggered exocytosis by measuring changes in whole terminal membrane capacitance (C_m) reflecting the increase in surface membrane area due to the fusion of vesicles.

With the whole-cell patch-clamp technique¹², we applied a sinusoidal voltage stimulus to the terminal, and analysed the membrane current at two orthogonal phase angles^{6,13-15} (Fig. 1). The C output indicated computed changes in C_m , and the G output indicated changes in both series resistance (R_s) and membrane conductance (G_m). A step depolarization activated inward Na^+ - and Ca^{2+} -currents⁸ (lower trace). On resuming sinusoidal excitation, the G signal was unchanged. By contrast, the first C computation indicated that a 'jump' increase of 80 femtofarads (fF) occurred during the pulse interval. Such an increase corresponds to the fusion of 20-80 peptidergic vesicles.

When the interval between depolarizations was > 8 s, capacitance jump and Ca^{2+} -current amplitudes were reproducible over several minutes. Figure 2a illustrates data from a terminal that showed an average increase of 48 fF when depolarized to +20 mV for 80 ms. During 13 min of recording from this terminal, 32 depolarizations to various potentials increased C_m by 957 fF. Such increases were not usually followed by decreases in C_m , although we have observed occasional rapid 'backsteps' (amplitude 5-40 fF) or slow relaxations of C_m , possibly reflecting endocytotic activity⁶.

Capacitance jump amplitudes were sensitive to the amount of calcium entry¹⁶ and calcium buffering^{6,17}. The peak of the voltage-dependence for increases in C_m occurred near +30 mV, corresponding to the maximum of the Ca^{2+} -current/voltage relation under the recording conditions that we used (Fig. 2b, c).

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Below the peak (where fewer calcium channels were activated), or above it (where the electrochemical driving force for Ca^{2+} entry was reduced), the smaller capacitance jumps indicate fewer vesicle fusions. In other terminals, depolarizations to the reversal potential of the macroscopic calcium conductance (about +70 mV) did not produce observable increases in capacitance (data not shown). This indicates that depolarization of the terminal membrane alone or Ca^{2+} entry during the tail current^{18,19} may not be sufficient to produce a secretory response under our conditions. Exocytosis also depended on the elevation of free intraterminal Ca^{2+} and not simply on increases in total calcium^{6,17}. For example, only 2 of 17 terminals perfused with

5 mM EGTA showed capacitance jumps in response to depolarizations, in contrast to 14 of 17 terminals perfused with 500 μM EGTA.

The rate at which depolarizing pulses were applied influenced the coupling between Ca^{2+} entry and capacitance response (Fig. 3). With repetitive-pulse protocols, the amplitude of the C jumps initially increased for successive pulses, usually peaking at the third pulse. This 'facilitation' occurred despite the reduction of peak inward Ca^{2+} -current (Fig. 3a, lower panels). The terminal then showed 'fatigue' or 'depression', because the amplitude of the C jumps for the fourth and fifth pulses was only 20–40% of the peak response. Recovery was relatively slow: 12 s later,

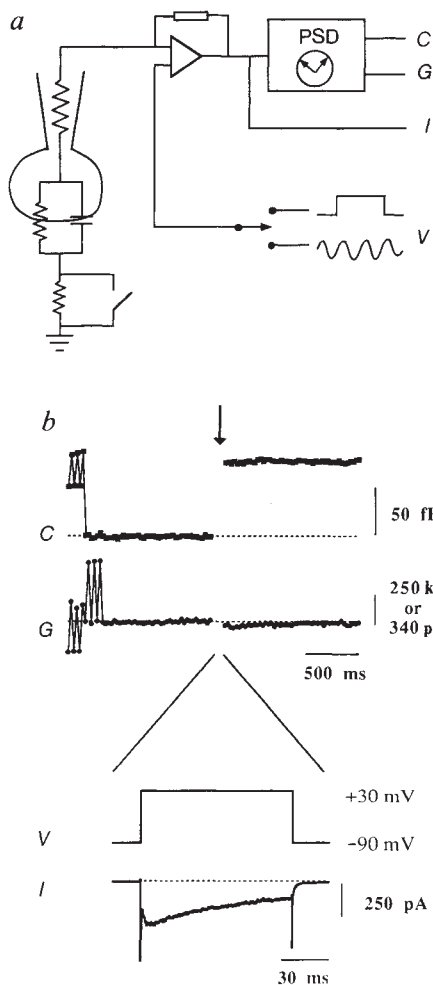


FIG. 2 Depolarization-triggered capacitance increases are reproducible and depend on Ca^{2+} entry. **a**, Three consecutive responses to 80-ms pulses to +20 mV. Left panel shows C records, where the break in each trace marks the pulse duration as in Fig. 1b. Right panel shows corresponding pulse currents. Interval between pulses, 8.5 s. Initial terminal capacitance = 5 pF, corresponding to 12.6 μm diameter, assuming spherical geometry. **b**, C jumps elicited by 80-ms pulses to the indicated membrane potential (mV). Same terminal as in Fig. 2a. **c**, Voltage dependence of the C jumps (upper) and peak current (lower) for this terminal. Each point on the ΔC_m - V plot shows the mean $\pm$ s.d. for three to five depolarizations. The points on the peak I_{Ca} - V plot show the raw current data.

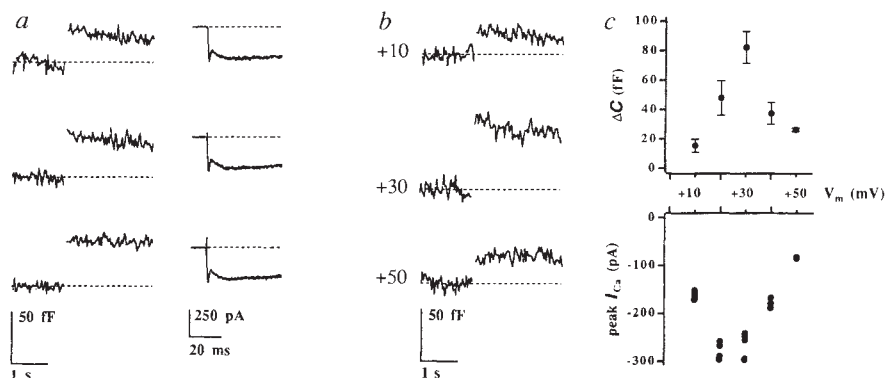
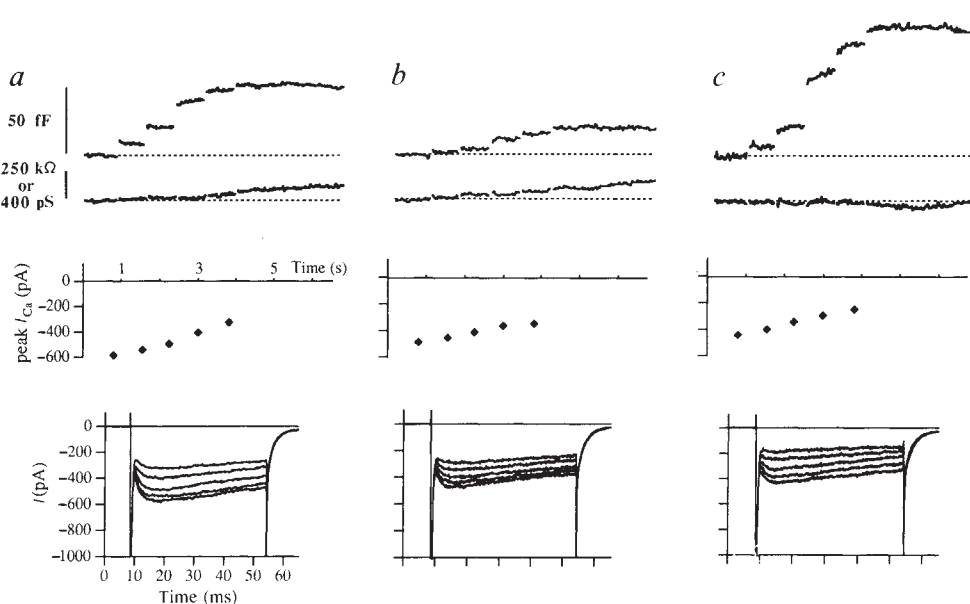


FIG. 1 Depolarization causes a rapid increase in nerve-terminal membrane surface area. **a**, Schematic illustration of the phase detection method⁶ for recording changes in the cell membrane capacitance C . V , applied membrane potential (sinusoidal stimulation or depolarizing pulses); I , resulting membrane current; G , combination of series resistance R_s and membrane conductance G_m . **b**, Top two recordings are the outputs of the phase-sensitive detector. Calibration: 50 fF for the C trace, and either 250 k Ω (R_s) or 340 pS (G_m) for the G trace. Arrow indicates application of an 80-ms depolarizing pulse to +30 mV from a holding potential of -90 mV (third trace). The resulting membrane current is shown in the lower trace: the initial large downward deflection is a tetrodotoxin sensitive Na^+ -current (I_{Na}) followed by a slowly decaying Ca^{2+} -current (I_{Ca}) resulting from the opening of two types of Ca^{2+} channels⁸. For this terminal, C_m = 2.4 pF, R_s = 5.7 M Ω , and G_m = 230 pS.

METHODS. Whole-terminal membrane current was recorded with an EPC-7 patch-clamp amplifier. To measure changes in cell membrane capacitance⁶, a 15-mV r.m.s. 1.2-kHz sine wave was added to the holding potential of -90 mV. The resulting current was analysed at two orthogonal phase angles, using a software-based phase-sensitive detector (PSD)¹⁴, with a temporal resolution of 28 ms per point. The correct phase angle for the PSD was located automatically with the 'phase tracking' technique¹⁵. In brief, a 500-k Ω resistor in series with the cell input impedance and ground was switched in and out during sinusoidal stimulation (vertical deflections at the start of C and G traces, Fig. 1b). The deflections that appeared only on the G trace when the resistor was switched on, served as a calibration of 500-k Ω changes in R_s . Correct alignment of the PSD is indicated by the lack of projection of these R_s changes on the C trace. C -trace calibration was obtained by unbalancing the C_{slow} compensation by 100 fF, after cancellation of whole-terminal capacitance with the C_{slow} network. Exocytosis was triggered by interrupting the sinusoidal stimulus and applying a depolarizing pulse of variable amplitude and duration. The potential was returned to -90 mV for 15–50 ms before re-starting the sine-wave stimulus. Kinetics of exocytosis could have been slower than normal, because the recording temperature was 22–25 $^{\circ}\text{C}$ and not 37 $^{\circ}\text{C}$. The large peptide-containing secretory vesicles have diameters of 175 to 350 nm estimated from electron micrographs²⁵ and quasi-elastic laser light scattering (B. Salzberg, personal communication), respectively. The expected increase in membrane area from a single vesicle fusion was 0.1–0.4 μm^2 , corresponding to C_m increases of 1–4 fF, assuming a specific C_m of 1 $\mu\text{F cm}^{-2}$. A 10- μm diameter terminal is expected to have ~30,000 secretory vesicles¹⁰. Peptide-releasing nerve terminals were isolated from rat neurohypophysis as previously described^{7,10}, except that suspensions of terminals were plated on poly-L-lysine-coated cover slips and maintained in DMEM medium (GIBCO) with 5% calf serum in a 37 $^{\circ}\text{C}$ incubator for 1–10 h before recording. Internal solution (mM): caesium glutamate (130), tetraethylammonium chloride (10), MgCl_2 (1), HEPES buffer (20), Mg-ATP (2), EGTA (0.5), cAMP (0.125), pH 7.25. External solution (mM): N -methyl-D-glucamine chloride (125), NaCl (25), KCl (5), CaCl_2 (10), MgCl_2 (1), glucose (10), HEPES buffer (10), pH 7.25. Holding current was adjusted to 0 with the pipette in the bath. No further correction was made for a junction potential introduced by these solutions.

FIG. 3 Repetitive-pulse protocols reveal facilitation, fatigue, and recovery of the release process. *a*, Terminal was depolarized to +30 mV with five 45-ms pulses at intervals of 750 ms. The top panel shows resulting capacitance (upper) and conductance (lower) responses. The peak I_{Ca} measured during each pulse is plotted in the middle panel with the same time base as the *C* and *G* traces. The full current records for the series of five pulses are superimposed in the lower panel. *b*, Same pulse protocol applied 12 s after that of *a*; capacitance increase in *b* was 37% of the increase in *a*, despite 82% recovery in I_{Ca} . *c*, Traces were recorded 1.2 min later than those of *b* and showed larger total C_m increase (205 fF) than those of *a* (111 fF). Drift in *G* traces probably reflected small increases in R_s which introduce a $<1^\circ$ change in the phase angle and $<0.5\%$ error in *C*-amplitude estimation. Initial terminal capacitance, 3.6 pF.



a pulse train elicited only small capacitance jumps, despite recovery of Ca^{2+} -currents. A robust response returned after a prolonged rest (>1 min, Fig. 3c). Seven of nine terminals examined with repetitive-pulse protocols showed similar patterns of facilitation and fatigue, and required a recovery period longer than the time necessary for removal of Ca^{2+} -current inactivation.

These data demonstrate that capacitance responses to membrane depolarization can be measured in single neurohypophyseal nerve endings under whole-terminal clamp and perfusion. The observed increases in C_m required Ca^{2+} entry and were modulated by Ca^{2+} buffering. The importance of steps after Ca^{2+} entry is indicated by the experiments using repetitive-pulse protocols. The observed facilitation could result from a build-up in the concentration of free intracellular calcium ($[Ca^{2+}]_i$)^{20,21}, whereas attenuation could reflect depletion of a pool of vesicles capable of being released, or inactivation of the release-site machinery²². These hypotheses about the sequential reactions of excitation-secretion coupling can be tested by combining capacitance-measuring techniques with a variety of cellular and molecular probes^{16,23,24}. □

Cardiac-type excitation-contraction coupling in dysgenic skeletal muscle injected with cardiac dihydropyridine receptor cDNA

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THERE are dihydropyridine (DHP)-sensitive calcium currents in both skeletal and cardiac muscle cells, although the properties of these currents are very different in the two cell types¹ (for simplicity, we refer to currents in both tissues as L-type²). The mechanisms of depolarization-contraction coupling also differ. As the predominant voltage-dependent calcium current of cardiac cells¹, the L-type current represents a major pathway for entry of extracellular calcium. This entry triggers the subsequent large release of calcium from the sarcoplasmic reticulum (SR)³⁻⁵. In contrast, depolarization of skeletal muscle releases calcium from the SR^{6,7} without the requirement for entry of extracellular calcium through L-type calcium channels^{8,9}. To investigate the molecular basis for these differences in calcium currents and in excitation-contraction (E-C) coupling, we expressed complementary DNAs for the DHP receptors from skeletal¹⁰ and cardiac muscle¹¹ in dysgenic skeletal muscle. We compared the properties of the L-type channels produced and showed that expression of a cardiac calcium channel in skeletal muscle cells results in E-C coupling resembling that of cardiac muscle.

Recently, we showed that E-C coupling and slow L-type calcium current, which are both missing in skeletal muscle of mice with muscular dysgenesis^{12,13}, are restored by the injection of an expression plasmid (pCAC6) containing a cDNA for the skeletal muscle DHP receptor¹⁴. Here, we injected an expression plasmid (pCARD1)¹¹, carrying the entire protein-coding sequence of the rabbit cardiac DHP receptor cDNA, into nuclei

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of dysgenic myotubes in primary culture. Both the pCAC6-injected¹⁴ and the pCARD1-injected dysgenic myotubes displayed spontaneous and electrically evoked contractions. Approximately 530 myotubes survived injection with pCARD1; of these, depolarization-contraction coupling was restored in 89, with 10 contracting spontaneously, and 79 in response to electrical stimulation. Of the roughly 180 myotubes that survived injection with pCAC6, E-C coupling was restored in 68 cases (17 spontaneous and 51 electrically evoked).

Dysgenic myotubes that had been injected with pCARD1 and observed to contract expressed an L-type calcium current whose kinetics and voltage-dependence were very different from those of the L-type calcium current in pCAC6-injected myotubes (Fig. 1). In pCAC6-injected myotubes, the rate of activation was slow, similar to that observed in normal skeletal muscle^{15,16}. In contrast, the L-type calcium current in pCARD1-injected myotubes was activated more rapidly, like the L-type current in cardiac muscle^{17,18}. The maximum value of the L-type current in pCARD1-injected myotubes was substantially larger than in pCAC6-injected myotubes (see Fig. 1 legend). This is partly because the maximal current in pCARD1-injected myotubes is

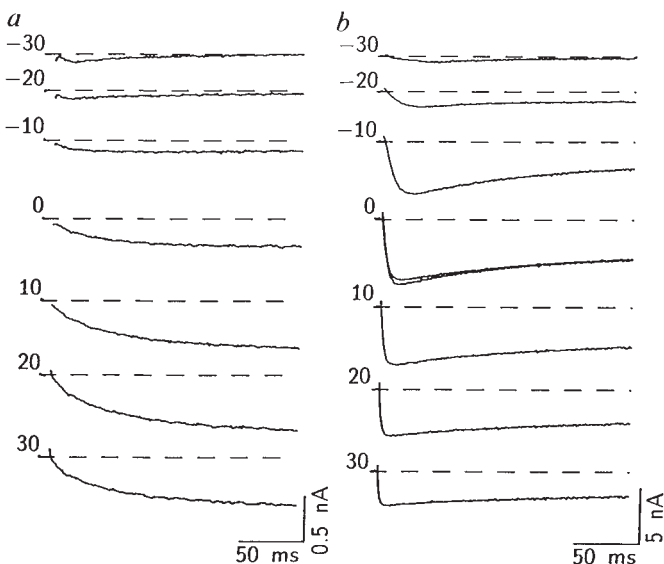


FIG. 1 Comparison of calcium currents in dysgenic myotubes that were injected with expression plasmid pCAC6 containing a cDNA for the skeletal muscle DHP receptor (a) or pCARD1 with a cDNA for the cardiac DHP receptor (b). Calcium currents were measured using the whole-cell variant of the patch-clamp technique²⁵. The test potential (in mV) is indicated next to each current trace. For both the cells shown, only a small amount of I_{fast} (T-type calcium current²) was present. The contribution of I_{fast} to the total current in the pCARD1-injected cell can be seen by comparing the two current traces for the 0 mV test potential. The larger current trace was obtained from the standard holding potential (-80 mV for all the data reported here). The smaller current trace was obtained for an identical 0 mV test pulse that was preceded by a 1 s step to -40 mV, which selectively inactivates I_{fast} . The average maximum L-type current (normalized by linear cell capacitance) was 4.4 ± 2.7 pA/pF (mean $\pm$ s.d., range 1.0–12.4 pA/pF; $n=26$) in pCAC6-injected myotubes and 28.0 ± 10.0 pA/pF (range 12.4–56.1 pA/pF; $n=31$) in pCARD1-injected myotubes.

METHODS. The procedures for preparation of primary cultures of dysgenic myotubes, for injection of the expression plasmid, for monitoring of contraction and for measurement of whole-cell currents were as previously described¹⁴, except that the concentration of the DNA injected was reduced by half. Test currents were corrected for linear components of capacitive and leak currents by digital scaling and subtraction of a control current elicited by a 30 mV hyperpolarization from the holding potential. This control current was also used to calculate the linear capacitance (C) for each cell. a, cell I66, $C=210$ pF; b, cell I69, $C=220$ pF. The pipette solution contained (in mM): 140, caesium aspartate; 5, MgCl₂; 10, Cs₂EGTA; 10, HEPES (pH 7.4 with CsOH). The bathing solution contained (in mM): 10, Ca²⁺; 145, tetraethylammonium ion; 165, Cl⁻; 0.003, tetrodotoxin; 10, HEPES (pH 7.4 with CsOH).

activated at a potential which is about 40 mV more negative; at this potential, the driving force on calcium is greater. Differences in the levels of channel expression, in single-channel conductance, or in opening probability, probably also contribute to the difference in maximal current.

In cardiac cells, the substitution of Ba²⁺ for Ca²⁺ produces a large increase in peak L-type calcium current¹⁹, whereas this same substitution produces a much smaller increase in the L-type calcium current of mammalian skeletal muscle²⁰. Figure 2 shows that Ba²⁺ substitution produced similar differential effects on the two channel types expressed in dysgenic myotubes. In cardiac cells, the substitution eliminates the prominent inactivation seen with Ca²⁺ as the charge carrier¹⁹. This was not the case when the cardiac channel was expressed in dysgenic myotubes, where the currents carried by Ca²⁺ and Ba²⁺ had similar kinetics. Previous studies have suggested that the DHP receptor alone is sufficient to produce L-type calcium channel activity^{11,21}, but additional subunits that are not produced in skeletal muscle might be necessary for the Ca²⁺-dependent inactivation of the cardiac L-type current.

The dihydropyridine (+)PN 200-110 (500 nM) blocked the L-type calcium current of both pCAC6-injected and pCARD1-injected dysgenic myotubes. Measured from a holding potential of -80 mV, the calculated half-blocking concentration (assuming that a single drug molecule blocks) was 137 ± 70 nM (mean $\pm$ s.d., $n=10$) and 235 ± 81 nM ($n=8$) for the cardiac and skeletal muscle channels, respectively. Both types of channels are blocked by more than 90% by 0.5 mM Cd²⁺.

Although the injection of either pCAC6 or pCARD1 restored depolarization-contraction coupling in dysgenic myotubes, the nature of this coupling was different for the two kinds of DHP receptor. Dysgenic myotubes injected with pCAC6 underwent electrically evoked contractions in normal rodent Ringer's sol-

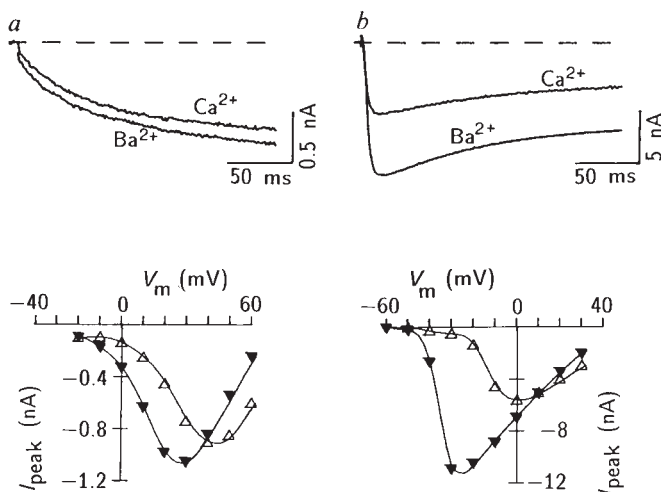


FIG. 2 Effect of substituting Ba²⁺ for Ca²⁺ on the L-type calcium current in dysgenic myotubes that were injected with pCAC6 (a) or pCARD1 (b). For each cell the maximum current recorded with Ca²⁺ (test potentials of +40 and 0 mV, respectively, in a and b) is illustrated for comparison with the maximum current recorded with Ba²⁺ (test potentials of +30 and -30 mV, respectively, in a and b). For the current traces illustrated in a, the test pulse was preceded by a 1 s pulse to -40 mV to inactivate I_{fast} which was quite large in this cell relative to the L-type current. The peak current (I_{peak}) is plotted against test potential (V_m) beneath the current traces for each cell. Data obtained with Ca²⁺ as charge carrier are indicated by open triangles, and data with Ba²⁺ by closed inverted triangles. For the cell shown in a, the peak L-type current was estimated as the current remaining at the end of the 200 ms test pulse for potentials ≤ 10 mV.

METHODS. The Ba²⁺-containing bathing solution was prepared by substituting 10 mM Ba²⁺ for 10 mM Ca²⁺ in the bathing solution. a, cell I89, $C=235$ pF; b, cell I69, $C=220$ pF. The average peak $I_{\text{Ba}}/I_{\text{Ca}}$ was 1.24 ± 0.09 ($n=3$) in pCAC6-injected myotubes and 1.92 ± 0.23 ($n=4$) in pCARD1-injected myotubes.

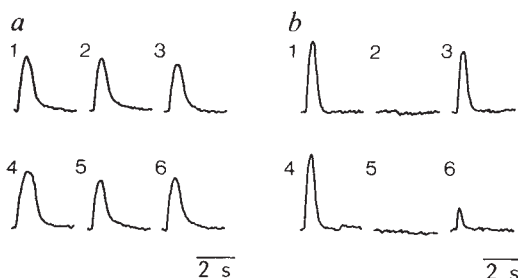


FIG. 3 Comparison of electrically evoked contractions in dysgenic myotubes that were injected with pCAC6 (a) or pCARD1 (b). The cells were first bathed in normal rodent Ringer (traces 1, 4), then exposed to either Ca^{2+} -free (trace 2) or 0.5 mM Cd^{2+} -containing Ringer (trace 5), and finally returned to normal Ringer (traces 3, 6). The recovery of electrically evoked contraction in the pCARD1-injected cell after exposure to Cd^{2+} was incomplete during the ~ 5 min wash with normal Ca^{2+} -free Ringer. However, after being returned to culture medium and maintained for ~ 30 min in the tissue culture incubator, the cell regained the ability to contract vigorously. The blockade of contraction in pCARD1-injected myotubes by brief exposures to lower concentrations of Cd^{2+} was readily reversible.

METHODS. The methods for electrical stimulation and optical recording of contraction were as described previously¹⁴. Electrical stimulation was applied ~ 150 ms after the onset of each trace illustrated. The normal rodent Ringer contained (in mM): 146, NaCl; 5, KCl; 2, CaCl_2 ; 1, MgCl_2 ; 10, HEPES (pH 7.4 with NaOH). The Ca^{2+} -free Ringer was made by equimolar substitution of Mg^{2+} for Ca^{2+} in the normal rodent Ringer. Vertical scale, arbitrary units.

ution (Fig. 3a, traces 1 and 4), in Ca^{2+} -free Ringer (Fig. 3a, trace 2) and in 0.5 mM Cd^{2+} -containing Ringer (Fig. 3a, trace 5), as is the case for normal myotubes in which Ca^{2+} entering across the sarcolemma is not necessary for E-C coupling⁹. On the other hand, pCARD1-injected dysgenic myotubes displayed electrically evoked contractions in normal rodent Ringer (Fig. 3b, traces 1 and 4), but not in Ca^{2+} -free Ringer (Fig. 3b, trace 2) or in 0.5 mM Cd^{2+} -containing Ringer (Fig. 3b, trace 5). Thus, unlike the E-C coupling that is restored in dysgenic myotubes by injection of pCAC6, depolarization-contraction coupling restored in dysgenic myotubes by injection of pCARD1 requires the voltage-dependent entry of Ca^{2+} .

To examine whether Ca^{2+} -induced release of Ca^{2+} from the SR is involved in the contraction of pCARD1-injected myotubes, we measured calcium current and contraction simultaneously after treating the myotubes with caffeine to deplete the SR of Ca^{2+} (Fig. 4). The calcium currents were nearly identical for successive, identical depolarizations, whereas the contraction was initially weak but increased in strength for successive pulses, until the fifth depolarization when it reached an approximately constant size. A simple explanation for this behaviour is that, during each of the first few test depolarizations, most of the Ca^{2+} that entered, and most that was released by the SR, was recycled into the SR by active transport, so that the amount of Ca^{2+} available to be released increased until the SR was full. Essentially identical behaviour has previously been demonstrated for caffeine-treated cardiac myocytes⁵. Thus, it appears that depolarization-contraction coupling in pCARD1-injected dysgenic myotubes mimics coupling in cardiac cells: Ca^{2+} entry is required and the Ca^{2+} that enters triggers additional Ca^{2+} release from the SR.

Recently, it has been shown that dysgenic skeletal muscle contains a calcium current, termed $I_{\text{Ca-dys}}$ (ref. 22), with characteristics which resemble those of the L-type calcium current expressed in pCARD1-injected myotubes. However, in control dysgenic myotubes grown under the conditions used here, $I_{\text{Ca-dys}}$ is typically too small to be detectable²²; it is, on average, less than 2% of the L-type calcium current that we have found to be expressed in pCARD1-injected myotubes.

The skeletal muscle DHP receptor functions both as an essential component of E-C coupling and as an L-type calcium

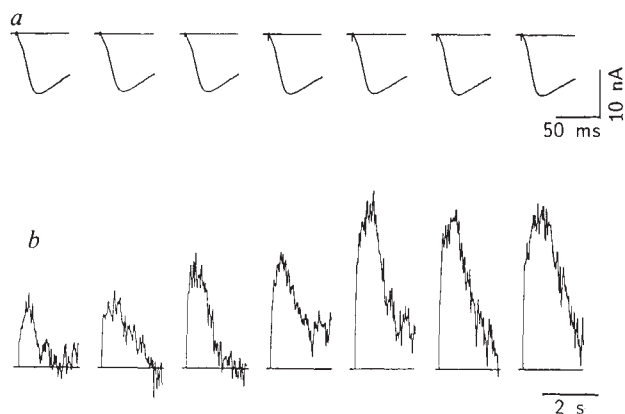


FIG. 4 Involvement of SR calcium release in contraction of a pCARD1-injected dysgenic myotube. The myotube was treated with caffeine to cause partial depletion of Ca^{2+} within the SR and then returned to a 2 mM Ca^{2+} -containing bath solution. To permit simultaneous measurement of calcium currents (a) and contractions (b), the EGTA content of the pipette solution was reduced to 0.1 mM. These conditions allowed stable recording of contractions from control pCARD1-injected myotubes. Calcium currents were elicited every 8 s by a 60 ms pulse to -10 mV. Currents and contractions are illustrated from left to right in the order in which they were obtained. Behaviour of calcium currents and contractions similar to that shown was observed in four other pCARD1-injected myotubes treated with caffeine.

METHODS. The bathing and pipette solutions were identical to those described in the Fig. 1 legend, except that in the bathing solution Ca^{2+} was reduced to 2 mM and Cl^- was reduced to 149 mM, and in the pipette solution $\text{Ca}_2\text{-EGTA}$ was reduced to 0.1 mM and caesium aspartate was increased to 155 mM. For the caffeine treatment, the culture dish was perfused (at ~ 6 ml min^{-1}) sequentially with 20 ml each of Ca^{2+} -free Ringer (composition given in Fig. 3 legend), Ca^{2+} -free Ringer containing 0.5 mM Na_2EGTA and 1 mM caffeine, Ca^{2+} -free Ringer containing 0.5 mM Na_2EGTA and 10 mM caffeine, and finally Ca^{2+} -free Ringer. Vertical scale in b, arbitrary units. Cell B46, $C = 795$ pF.

channel^{10,14}. In the former role, the skeletal muscle DHP receptor probably represents the voltage sensor^{10,14,23,24} that controls the release of Ca^{2+} from the SR in response to transverse tubular depolarization, without the need for entry of Ca^{2+} across the sarcolemma. The cardiac DHP receptor expressed in skeletal muscle is able to function as an L-type calcium channel, but not as a voltage sensor that directly controls release of Ca^{2+} from the SR. □

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Effect of cell history on response to helix-loop-helix family of myogenic regulators

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IN multinucleated heterokaryons formed from the fusion of differentiated muscle cells to either hepatocytes or fibroblasts, muscle-specific gene expression is activated, liver-specific gene expression is repressed, and there are changes in the location of the Golgi apparatus¹⁻³. An understanding of the regulatory mechanisms that underlie this plasticity is of particular interest given the stability of the differentiated state *in vivo*. We have now investigated whether MyoD or myogenin, regulators of muscle-specific gene expression that have a helix-loop-helix motif⁴⁻⁷, can induce the phenotypic conversion observed in heterokaryons. When these regulators were stably or transiently introduced into fibroblasts or hepatocytes by microinjection, transfection or retroviral infection with complementary DNA in expression vectors, fibroblasts expressed muscle-specific genes, whereas hepatocytes did not. However, fusion of hepatocytes stably expressing MyoD to fibroblasts resulted in activation in the heterokaryon of muscle-specific genes of both cell types. These results imply that other regulators, present in fibroblasts but not in hepatocytes, are necessary for the activation of muscle-specific genes, and indicate that the differenti-

ated state of a cell is dictated by its history and a dynamic interaction among the proteins that it contains.

To determine whether MyoD or myogenin is sufficient to induce the expression of the muscle phenotype, we introduced these regulators into different cell types that in heterokaryons activate and express muscle-specific genes². These include primary human lung fibroblasts (MRC5), the human hepatocyte cell line HepG2, which stably expresses 50 liver-specific functions, and primary human hepatocytes isolated directly from fetal liver^{8,9}. For reference, we compared the effects of MyoD and myogenin in these cell types with those in NIH3T3 and 10T1/2 fibroblasts, which show a high frequency of muscle gene expression in response to these regulators^{4,6}. Below we describe the results obtained with MyoD, but as indicated in Table 1, myogenin, although less efficient, yielded essentially similar results to those with MyoD, and was not synergistic when combined with MyoD.

To assess the short-term effects of MyoD, we monitored muscle gene expression in transient assays. We introduced MyoD cDNA by microinjection, because the cell types analysed differed 100-fold in transfection efficiency. This method permitted a direct assessment of the proportion of cells in which muscle genes were activated when exposed to MyoD (Fig. 1; Table 1, A). Moreover, by using this approach, we could analyse cell types that showed limited proliferation, such as primary

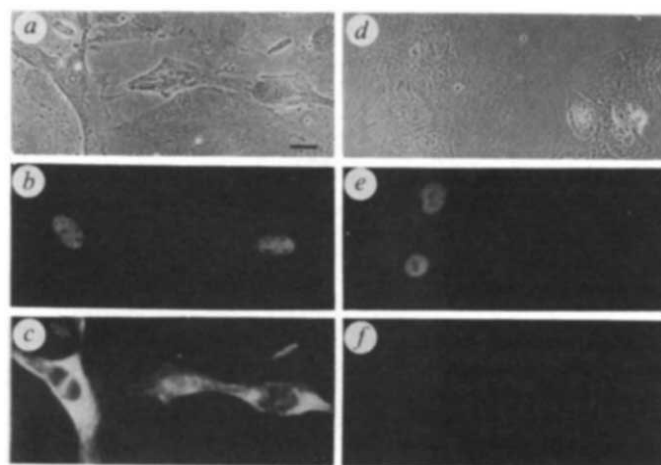


FIG. 1 Microinjection of MyoD cDNA under the control of the MSV LTR leads to activation of muscle genes in NIH3T3 cells (left) but not in primary hepatocytes (right). Injected cells, shown in phase contrast (a, d), were identified by including a fluorescent dye (FITC-dextran, 25 mg ml⁻¹, Molecular Probes) in the sample (b, e), and were assayed 3 days after injection for the expression of myosin heavy chain by immunofluorescence (c, f). MyoD cDNA⁴ was injected into the nucleus at a concentration of 0.5 mg ml⁻¹ in 10 mM Tris-HCl buffer, 0.1 mM EDTA (pH 7.4) using a microinjector (Eppendorf 5242). Cells were grown in DMEM containing 10% supplemented calf serum, except for primary hepatocytes, which were grown as described previously⁹. All cells were switched to differentiation medium (DMEM containing 2% horse serum) 12 h before, and continuously after, microinjection to promote muscle differentiation and diminish proliferation. Cells were fixed as previously described²⁸, then reacted with monoclonal antibody 4A.1025 and rhodamine-conjugated goat anti-mouse IgG (Cappel; 1:100), each for 30 min at 37 °C to reveal myosin heavy chain¹⁰. A Zeiss Axiophot microscope was used to score and photograph cells (bar, 20 µm).

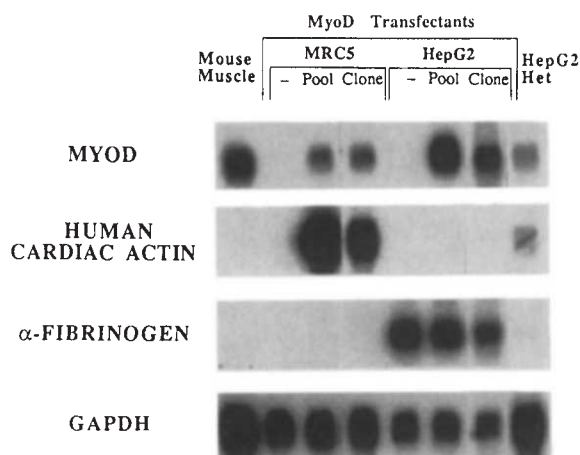


FIG. 2 MyoD mRNA is expressed by stably transfected MRC5 and HepG2 cells. Total RNA from MRC5 and HepG2 cells stably transfected with MSV/myoD was hybridized sequentially to probes for MyoD, human muscle α -cardiac actin, liver α -fibrinogen, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Shown here are MRC5-myoD clone 5 and HepG2-myoD clone 3 from Table 1, C; the minus sign designates untransfected controls. The low level of human cardiac actin transcripts in heterokaryons (HepG2 het) reflects the fact that only 16% of all nuclei were HepG2 nuclei inside heterokaryons. GAPDH serves as a marker for comparing mRNA levels in diverse cell types²⁹. Total RNA was isolated from cells by lysis in guanidine thiocyanate followed by pelleting of the RNA through CsCl. Mouse muscle RNA was from C2C12 cells cultured for one day in differentiation medium. Heterokaryon RNA was isolated 4 days after fusion. RNA (10 µg per lane) was electrophoresed on a formaldehyde gel and blotted onto nylon membrane (Nytran) according to the manufacturer (Schleicher and Schuell). Complementary DNA probes used were a 1.8-kilobase (kb) EcoRI fragment for MyoD⁴, a 1.7-kb PstI fragment for α -fibrinogen³⁰, a 1.2-kb EcoRI fragment for GAPDH³¹, and a 101-bp Ddel-RsaI fragment which is specific for human α -cardiac actin³². DNA probes were made by extension of random hexamers, except for human α -cardiac actin, for which an RNA-probe was generated. Specific activities for all probes were greater than 10⁸ c.p.m./µg⁻¹. Hybridization was for 24 h in 50% formamide, 5 × SSPE, 5 × Denhardt's solution, 1% SDS, 200 µg ml⁻¹ sheared calf thymus DNA, 200 µg ml⁻¹ yeast transfer RNA, 50 µg ml⁻¹ poly(A) at 42 °C (60 °C for human α -cardiac actin). The blots were washed three times for 20 min each in 0.1 × SSPE, 1% SDS at 65 °C, and then exposed to film with an intensifying screen. The MyoD film shown was exposed for 12 h; human α -cardiac actin for 25 h; α -fibrinogen for 454 h; GAPDH for 58 h. Probes were removed by washing the blot in 50% formamide, 5 × SSPE at 65 °C for 1 h.

TABLE 1 Microinjection, retroviral infection and transfection data

A. Microinjection of MyoD (transient expression)

Expression of muscle myosin heavy chain (3 days)

	MSVmyoD % (n)	RSVmyoD % (n)	MSVmyogenin % (n)	MSVmyoD and MSVmyogenin % (n)	Control pSV2CD8 % (n)
NIH3T3	61 (115)	66 (17)	11 (35)	65 (83)	80 (64)
10T1/2	60 (118)	52 (93)	12 (32)	51 (47)	80 (49)
MRC5	0 (181)	0 (227)	0 (56)	0 (93)	80 (41)
HepG2	0 (267)	0 (124)	0 (66)	0 (120)	52 (56)
Primary hepatocytes	0 (135)	0 (90)	0 (88)	0 (74)	73 (41)
HepG2-MyoD clone 3	—	—	0 (97)	—	—

Expression of liver functions

	Albumin		Fibrinogen	
	MSVmyoD % (n)	RSVmyoD % (n)	MSVmyoD % (n)	RSVmyoD % (n)
HepG2	100 (68)	100 (76)	100 (46)	100 (61)
Primary hepatocytes	87 (77)	88 (56)	82 (74)	90 (39)

B. Retroviral infection of MyoD

Expression of muscle myosin heavy chain

	4 days %	8 days %	12 days %
NIH3T3	44 ± 3	98 ± 1	—
10T1/2	37 ± 3	94 ± 1	—
MRC5	3 ± 1	78 ± 1	—
HepG2	0	0	0
Primary hepatocytes	0	0	0

C. Transfection of MyoD (stable expression)

Expression of muscle functions

	N-CAM %	Myosin %	Actin %	Golgi [†] %
MRC5 (untransfected)	0*	0	0	12 ± 3
MRC5-MyoD (pool)	16 ± 1	12 ± 1	17 ± 1	80 ± 3
MRC5-MyoD clone 1	43 ± 2	11 ± 2	12 ± 2	
clone 2	1 ± 1	1 ± 1	1 ± 1	
clone 3	15 ± 2	16 ± 2	15 ± 2	
clone 4	19 ± 2	19 ± 3	32 ± 3	
clone 5	6 ± 1	4 ± 1	10 ± 1	
HepG2 (untransfected)	0	0	0	8 ± 2
HepG2-MyoD (pool)	0	0	0	
clone 1–4	0	0	0	8 ± 1

Expression of liver functions

	Albumin %	Fibrinogen %	α_1 -Antitrypsin %
HepG2 (untransfected)	99 ± 1	96 ± 1	99 ± 1
HepG2-MyoD (pool)	99 ± 1	97 ± 1	99 ± 1
HepG2-MyoD clone 1	98 ± 1	86 ± 3	99 ± 1
clone 2	100	97 ± 1	100
clone 3	98 ± 1	99 ± 1	100
clone 4	96 ± 1	87 ± 3	99 ± 1

A. Microinjection: cells were microinjected with MyoD or myogenin cDNA and analysed 3 days later for expression of myosin heavy chain, as described in Fig. 1. Expression vectors included mouse MyoD cDNA driven either by the MSV LTR (MSVmyoD)⁴ or by the RSV LTR (RSVmyoD)¹⁵, and the mouse myogenin cDNA driven by the MSV LTR (MSVmyogenin)⁶, which were either injected alone or together (0.5 mg ml⁻¹ each). The frequency of myosin heavy-chain expression was corrected for the proportion of microinjected cells (fluorescein isothiocyanate (FITC) – dextran-positive nuclei) that expressed a control construct: a cDNA encoding a lymphocyte cell surface antigen, CD8 (ref. 21), driven by the simian virus 40 early promoter, and assayed by immunofluorescence²². Similar results were obtained when the DNA concentration was increased from 0.5 to 2.0 mg ml⁻¹ and the cells were assayed 3 or 6 days after injection. The proportion of primary hepatocytes that did not express liver functions reflects the nonhepatic cell population. **B. Retroviral infection:** cells were infected with an amphotropic retrovirus that expresses MyoD from the Moloney leukaemia virus LTR (LMDSN)¹³. Cells were infected with 10⁶ colony-forming units (c.f.u.) per dish, and expression of myosin heavy chain was assayed 4, 8 or 12 days later. The frequency of myosin heavy-chain expression was corrected for the infection frequency, determined by assaying either for β -galactosidase or MyoD after infection with the BAG (ref. 23) or LMDSN retroviruses, respectively, both of which were produced with packaging cell line PA317 (ref. 13). Cells were infected as described previously¹³. Between 10⁶ and 10⁷ hepatocytes were analysed at each time point. Error values, s.e. of the proportion. **C. Stable transfection:** HepG2 and MRC5 cells were co-transfected with MSVmyoD and plasmid pSV2neo and analysed either as pools or individual clones after selection. MRC5 fibroblasts were incubated with MSVmyoD (30 μ g) and pSV2neo (3 μ g) in DMEM medium, 10% calf serum and polybrene (15 μ g ml⁻¹) for 10 h and then exposed to dimethyl sulphoxide (30%) in DMEM for 4 min at room temperature. HepG2 cells were incubated with MSVmyoD (30 μ g) and pSV2neo (0.6 μ g) in calcium phosphate for 20 h and then in glycerol (15%) in HEPES-buffered saline for 3 min. Transfected MRC5 and HepG2 cells were grown in DMEM, 15% calf serum, 5% fetal calf serum, basic fibroblast growth factor (2.5 ng ml⁻¹; Amgen) and G418 (400 μ g ml⁻¹), and then exposed to a muscle differentiation medium for 4 days. Albumin, fibrinogen and α_1 -antitrypsin were detected on fixed cells with polyclonal antibodies (1:1,000; Cappel). N-CAM was detected with monoclonal antibody 5.1H11 (refs 24, 25), sarcomeric actin was detected with monoclonal antibody B4 (ref. 26), and galactosyl transferase of the Golgi was detected with polyclonal antibody O₁₂ (1:25 dilution)²⁷ as described previously³, except that saponin (0.1%) was used throughout in place of Tween-20. Error values, s.e. of the proportion.

* In each case, 0% indicates that no positive cells were seen on scanning 35–40 fields of at least 300 cells per field (>10,000 cells).

† The percentage of cells given is that which had a circumnuclear Golgi distribution typical of muscle. For the MRC5-MyoD pool, this value is the percentage of N-CAM-positive cells with a circumnuclear Golgi.

hepatocytes. To control for the differences in promoter strength that could arise as a result of diverse cell backgrounds, we used two different MyoD expression vectors, driven by either the Moloney sarcoma virus (MSV) long terminal repeat (LTR) or the Rous sarcoma virus (RSV) LTR. After a 3-day period, more than half of the injected NIH3T3 and 10T1/2 cells showed a dramatic change in cell shape visible by phase-contrast microscopy, and expressed sarcomeric actins and myosin heavy chains detectable by immunofluorescence microscopy (Fig. 1a). Expression of muscle functions was not observed in MRC5 fibroblasts, HepG2 liver cells or primary hepatocytes. The complete absence of myosin heavy-chain expression in MyoD-injected MRC5 fibroblasts is in marked contrast to the 50% of heterokaryons that expressed myosin at an equivalent time point after fusion of MRC5 fibroblasts to muscle cells¹⁰. Primary hepatocytes and HepG2 cells were indistinguishable: muscle genes were activated in heterokaryons with a similar frequency that was dependent on gene dosage (Fig. 3a), but were not activated in response to MyoD in transient assays (Table 1, A).

We designed two experiments to determine whether prolonged exposure to MyoD was required for MRC5 fibroblasts and liver cells to express muscle genes. First, we infected cells with a MyoD-expressing retrovirus that integrates into genomic DNA more frequently than does microinjected DNA and is therefore retained for longer periods of time (Table 1, B). Second, we transfected cells and obtained clones that stably expressed MyoD cDNA driven by the MSV LTR (MSVmyoD). These clones were designated MRC5-MyoD and HepG2-MyoD (Table 1, C). We assessed the expression of three myogenic proteins (human) neural cell-adhesion molecule (N-CAM), myosin heavy chain, and sarcomeric actin) and three liver proteins (albumin, fibrinogen and α_1 -antitrypsin), and the location of

the Golgi apparatus by immunofluorescence microscopy (Table 1, B and C). Both infected and stably transfected MRC5 fibroblasts showed, with varying frequency, all of the characteristics of muscle cells that we tested. However, the time required for muscle gene activation was longer in MRC5 fibroblasts than in NIH3T3 or 10T1/2 fibroblasts, which could reflect a requirement for either a higher threshold concentration of MyoD or induction of a regulator already present in NIH3T3 and 10T1/2 cells, possibly a repressor of a tissue-specific extinguisher like *tse-1* (ref. 11). In contrast to fibroblasts, neither primary hepatocytes nor HepG2 cells showed any of the changes observed in heterokaryons formed from fusion of these cells to muscle cells³: muscle-specific gene expression was not activated, liver-specific gene expression was not repressed, and the Golgi remained polar at one side of the nucleus.

Our next experiment ruled out the possibility that stably transfected liver cells fail to express muscle functions because they do not produce MyoD. We isolated total messenger RNA from individual clones and pools of clones of MRC5-MyoD and HepG2-MyoD. All of the transfected cells of Fig. 2 produced MyoD transcripts, but only the fibroblasts expressed human muscle cardiac actin transcripts. Moreover, although the level of MyoD transcripts in hepatocytes was higher than that in fibroblasts, and comparable to that in muscle cells, hepatocytes continued to express liver fibrinogen transcripts and did not express muscle transcripts. Individual stably transfected clones, such as MRC5-MyoD clone 5, that grew sufficiently well to permit mRNA isolation, generally expressed muscle gene products at a reduced level (Fig. 2) and frequency (Table 1, C) relative to pools of clones. This is presumably because MyoD expression is antagonistic to proliferation⁴, and pools of stably transfected MRC5 clones contained a mixture of cells with a

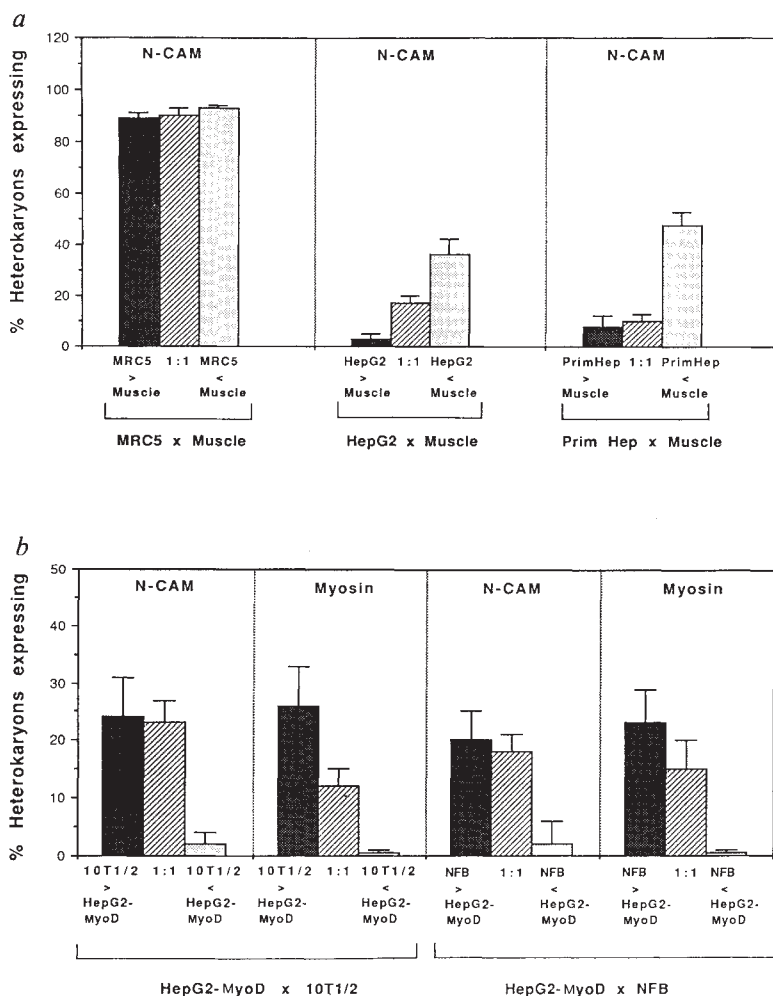


FIG. 3 Activation of muscle genes in heterokaryons requires regulatory factors in addition to MyoD and is dependent on gene dosage. Four days after fusion, heterokaryons were analysed for expression of human muscle N-CAM and myosin heavy chain, and were grouped into three categories according to the ratio of nuclei contributed by the two cell types. *a*, human N-CAM expression in primary hepatocytes and HepG2 cells, in contrast to MRC5 fibroblasts, depends on nuclear ratio. The frequency of muscle gene expression was lowest when the proportion of hepatocyte nuclei was increased, a pattern that did not change with time. *b*, N-CAM (human) and myosin heavy chain (mouse and human) expression were detected in heterokaryons formed from the fusion of HepG2-MyoD clone 3, which expresses MyoD (Fig. 2), to 10T1/2 fibroblasts or the myogenic variant NFB, neither of which express MyoD. Heterokaryons formed from fusion of untransfected HepG2 cells to 10T1/2 or NFB cells do not show activation of muscle genes (data not shown). Heterokaryons were produced as previously described¹ and assayed for expression of human N-CAM or mouse and human myosin heavy chain as described in Fig. 1 and Table 1. To determine nuclear ratio, the fluorescent dye Hoechst 33258 was used to distinguish mouse (punctate stain) and human (uniform stain) nuclei, as previously described⁴. Error bars, s.e. of the proportion. Prim Hep, primary hepatocytes.

range of growth potential and muscle gene expression. Similarly, pools of HepG2-MyoD cells expressed 5- to 10-fold more MyoD than did a clone such as HepG2-MyoD clone 3. The accumulation of MyoD protein in HepG2 cells paralleled that of MyoD transcripts. Each of the four stably transfected HepG2 clones of Table 1C produced MyoD protein in amounts comparable to those in muscle cells, as determined by immunoprecipitation (Fig. 4), but none expressed endogenous muscle genes.

The most compelling evidence that MyoD synthesized by clones such as HepG2-MyoD clone 3 is sufficient for myogenesis was based on function. When we fused HepG2-MyoD clone 3 cells to either mouse 10T1/2 fibroblasts or the myogenic variant NFB (ref. 34), which do not express MyoD, both the mouse and the human myogenic programmes were activated (Fig. 3b). Whereas the myosin heavy chain detected could have been produced by either cell type, the human muscle N-CAM could only have been produced by the HepG2 cells. The expression of muscle products was most frequent when the number of fibroblast nuclei was greater than that of hepatocyte (MyoD-producing) nuclei in the heterokaryon. This indicates that in such fusions, the increase in MyoD is counterbalanced by the increase in HepG2 components that are inhibitory to myogenic activation. The presence of an inhibitor is further indicated by the observation that the gene dosage effect in hepatocyte heterokaryons, unlike that in fibroblast heterokaryons, remains constant with time^{3,12}. It seems unlikely that a twofold dilution of the inhibitor on fusion with fibroblasts led to muscle gene activation by MyoD, because HepG2 cells that expressed significantly greater amounts of MyoD than did clone 3 did not activate muscle genes. Instead, the results indicate that positive regulators produced by 10T1/2 or NFB cells are required to modify or act together with MyoD to induce myogenesis. Whether these regulators pre-existed in these two cell types or were induced by MyoD cannot be determined from these experiments. We conclude that presumptive positive regulators contributed by 10T1/2 and NFB cells act directly or indirectly to override negative regulators present in liver cells.

The experiments reported here demonstrate that the activation of muscle-specific genes of certain cell types in heterokaryons

cannot be attributed to the action of MyoD or myogenin alone or in combination. Additional regulators are clearly implicated for two types of liver cells, primary hepatocytes and the well-differentiated HepG2 line, which are endodermal in origin. But for NIH3T3, 10T1/2 and MRC5 fibroblasts, which, like muscle cells, derive from the mesodermal lineage, the activity of MyoD is sufficient for myogenic conversion.

Our results are consistent with those reported by Weintraub and colleagues^{4,13}, but our conclusions differ. As discussed below, MyoD has a range of effects on differentiated cell types, which may reflect their derivation. MyoD heritably converts mesodermal fibroblasts to muscle⁴. However, several types of cells, including endodermal HepG2 cells, do not respond to MyoD¹³. By contrast, the liver-derived cell line BNL (ref. 14) has been reported to express myosin heavy chain in the presence of MyoD, although the frequency of this event was not given¹³. The ability of BNL to respond more readily to MyoD could be because it is less differentiated than HepG2 and primary hepatocytes; BNL cells express no detectable albumin and only trace amounts of fibrinogen (B.T.B. and H.M.B., unpublished observations). Weintraub *et al.*¹³ and Lin *et al.*¹⁵ also reported activation of muscle gene expression in a few other non-mesodermal cell types. However, in both the neuroblastoma and melanoma cell types examined, although the MyoD construct remained present, muscle gene expression was transient, and endogenous developmental programs persisted¹³, indicating that a heritable phenotypic change, such as that seen with mesodermal fibroblasts, was not induced. Together these findings indicate that cells differ in their response to MyoD, and that these differences are likely to be dictated by the history of the cells.

How can a gene such as that encoding MyoD have different effects depending on the cell background in which it is expressed? That gene function is influenced by the cellular environment has been demonstrated for components of signal transduction pathways: the transfection of a serotonin receptor gene into NIH3T3 cells leads to an oncogenic rather than a neural response¹⁶, and the microinjection of a viral oncogene into prospective ectodermal tissue causes it to differentiate as mesoderm¹⁷. A mechanism by which MyoD or myogenin could have different effects in different cell types is indicated by recent findings that transcriptional regulators with 'leucine-zipper' or 'helix-loop-helix' motifs bind DNA sequences as heterodimers^{7,18}. These regulators and another muscle regulator, Myf-5 (ref. 19), share homology with the Myc family of proteins, contain the helix-loop-helix motif, and form heterodimers with the immunoglobulin gene enhancer-binding protein E12 *in vitro*²⁰. We postulate that the inability of MyoD or myogenin to activate the muscle phenotype when introduced into liver cells results from the formation of novel heterodimers in that cell type, which could act as negative rather than positive regulators. The response of a cell to a single regulator depends on its protein composition, which is determined by its heritage, a history of responses to cues during development. The phenotypic changes observed in hepatocytes in heterokaryons differ from the phenotypic changes observed in hepatocytes into which a single regulator has been introduced, because the former result from a complex interaction of proteins contributed by each cell type of the heterokaryon. We conclude that the apparently stable differentiated state of a cell is the product of a dynamic interaction among the proteins it contains. □

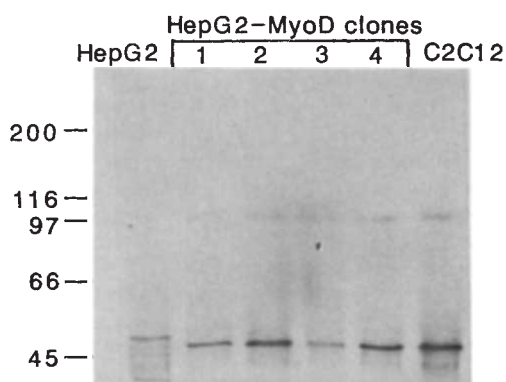


FIG. 4 Stably transfected HepG2 cells produce MyoD protein in amounts comparable to that of muscle cells. MyoD was immunoprecipitated from four different clones of HepG2 cells stably transfected with MSVmyoD (HepG2-MyoD clones 1-4 Table 1, C), from control untransfected HepG2 cells, and from C2C12 mouse muscle cells. Primary hepatocytes could not be stably transfected or infected at a sufficiently high frequency to permit an analysis of MyoD protein expression by immunoprecipitation. Cells were pulse-labelled with 125 μ Ci ml⁻¹ [³⁵S]methionine (1,100 Ci mmol⁻¹, Amersham) for 4 hours and processed as described by Tapscott *et al.*³³, using rabbit antisera to a MyoD fusion protein. Equal amounts of labelled protein, as determined by scintillation counting of trichloroacetic acid-precipitable material, were used in each immunoprecipitation. Immunoprecipitated material was electrophoresed on a 10% polyacrylamide gel. MyoD migrates with an apparent relative molecular mass (M_r) 45,000 to 48,000 (ref. 33) as indicated by M_r size standards ($M_r \times 10^{-3}$). Clones 3 and 4 were tested in heterokaryons with 10T1/2 cells, and both activated muscle genes.

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Expression and effects of the juvenile hormone esterase in a baculovirus vector

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THE endocrine changes that initiate the metamorphosis of insect caterpillars into pupae and, ultimately, into adult moths occur over a very short time period. Thus, it is not surprising that titres of the hormones that control this process seem to be regulated by changes in the rates of both biosynthesis and degradation. A reduction in the titre of juvenile hormone (JH) early in the last larval instar has been shown to initiate metamorphosis and lead to a cessation of feeding behaviour¹. This reduction in JH is associated with a dramatic increase in the levels of a very active, specific enzyme that hydrolyses the chemically stable, conjugated methyl ester to the biologically inactive JH acid². If this juvenile hormone esterase (JHE) is inhibited, the *in vivo* JH titre remains high enough to keep the larva in the feeding stage, resulting in giant insects³. Thus, if sufficient quantities of JHE were expressed *in vivo* at an early stage of development, the reduction in JH titre should cause the affected insect to stop feeding. Because of its low abundance, the purification of sufficient JHE from the blood of caterpillars was very difficult until the development of a highly efficient affinity purification system⁴. This system produced enough JHE for the development of antibodies and for partial amino-acid sequencing of JHE from several species including the major insect pest, *Heliothis virescens*^{5,6}. Three similar clones thought to encode JHE were obtained from a complementary DNA library made from fat bodies of *H. virescens*⁷. Here, we describe the expression of JHE in an *in vitro* baculovirus system which will provide enough enzyme for detailed biochemical and physiological studies. The results suggest that *in vivo* expression of JHE may help in the

improvement of genetically engineered viral insecticides which work by reducing insect feeding.

To confirm that the clones obtained previously actually encode JHE, it was important to demonstrate that the resulting proteins had catalytic activity when expressed *in vitro*. A baculovirus expression system which uses the moth *Autographa californica* nuclear polyhedrosis virus (AcNPV)⁸ was attractive for several reasons. The system is noted for its high levels of expression and for its efficient post-translational modification of many proteins^{9–12}. In the case of JHE, however, it offers additional advantages. AcNPV has a broad host range and infects a variety of noctuid larvae including the genera *Spodoptera*, *Trichoplusia* and, importantly, *Heliothis*. Thus, this system provided the opportunity to study the expression and post-translational modification of an insect enzyme in insect cells derived from species in the same family as *Heliothis*, as well as the possibility of testing the effects of the engineered virus in major insect pests *in vivo*.

The JHE gene was isolated from a Bluescript plasmid⁷ and inserted in the transfer vector pAcRP23 (refs 13, 14) to produce pAcRP23.JHE (Fig. 1). This plasmid was co-precipitated with AcNPV DNA and used to co-transfect cells of *Spodoptera frugiperda* (IPLB Sf 21)¹⁵. In all of the JHE transfections, the hydrolysis of JH was found to be both time-dependent and protein-dependent, whereas no hydrolysis of JH was observed with identical control transfections using the same transfer vector containing the *lacZ* gene. The recombinant virus was purified by five sequential plaque assays with screening for polyhedrin minus plaques using standard procedures⁸. At each stage, the presence of JHE in the plaque was confirmed by a JHE assay on enzyme eluted from agar above each plaque. The highly specific and sensitive JHE assay¹⁶ indicates that the JHE gene may provide a good reporter system for the study of baculovirus promoters. We used the standard esterase assay, except that 150 μ l of trichlorethylene was used instead of 250 μ l isooctane in the 1.5 ml polypropylene tubes.

In both monolayer and spinner flask culture of uninfected cells of *S. frugiperda*, JHE activity was not detectable by the standard assay. When the virus containing the JHE gene (AcRP23.JHE) was added (at 10 plaque-forming units per cell), the JHE activity in the medium began to increase rapidly at 10 h after infection, reaching a maximum at 48 h. The catalytic activity in the medium was, on average, about 100 nmol⁻¹ ml⁻¹ which is about twice the JHE activity seen at peak levels in blood obtained from *H. virescens*. This catalytic activity corresponds to about 75 mg of JHE per litre of medium. In serum-free medium, a single protein band was seen on sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) which co-migrated with the affinity-purified native protein. As expected from the sequence of the 19 amino-acid leader, deduced from the complementary DNA (cDNA) sequence of JHE, the protein seemed to be rapidly exported from *S. frugiperda* cells with 94–98% of the total catalytic activity appearing in the media when monitored at 24, 36, 48 and 72 h after infection.

The JHE activity in the medium surrounding infected *S. frugiperda* cells was compared, in several ways, with the activity in haemolymph collected from larvae of third or fourth instar *Trichoplusia ni* infected with the engineered virus (see below) and with the natural enzyme in *H. virescens* blood in the second day of the last larval instar. In each case, chromatographic analysis indicated that JH acid was the only metabolite produced from tritiated JH, and in each case hydrolysis was directly dependent upon incubation time and protein concentration over a wide range. The enzymes proved stable to repeated freeze thaw cycles, incubation at 5 or 30°C for several days, as well as to a variety of reagents. The natural and expressed enzymes were very resistant to eserine, a potent general esterase inhibitor, and to DFP (*O,O*-diisopropylphosphorofluoridate), a choline esterase inhibitor, but were inhibited by phenylmethyl sulphonyl

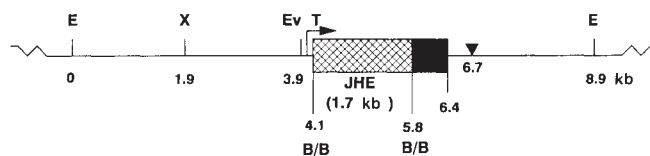
FIG. 1 Structure of the AcRP23.JHE virus. E, *EcoRI* sites; X, *XhoI* site; Ev, *EcoRV* site; B/B, *BamHI/BglII* ligation; T, transcription start site. The hatched area shows the coding region of JHE and the dark area the remaining polyhedron sequence.

METHODS. To prepare the transfer vector, the 3 kilobase (kb) JHE gene at the *EcoRI* cloning site of a Bluescript SK⁻ plasmid (plasmid 3hv16; ref. 7) was linearized by partial digestion with *EcoRI*. The ends were repaired with the Klenow fragment of *Escherichia coli* DNA polymerase and a *BglII* linker was placed at the 5' end of the JHE gene. After ligation and transformation the plasmid was linearized, again by partial digestion at a *Clal* site just downstream from the natural translation stop codon, and a second *BglII* linker was inserted at the 3' end of the gene (pJHE16B). This process resulted in the removal of the large non-coding 3' region of the JHE but did not remove the 1.7 kb coding sequence. The resulting plasmid was digested with *BglII* and the 1.7 kb JHE fragment isolated by agarose gel electrophoresis. The above procedure allowed the JHE gene to be inserted into a *BamHI* cloning site of the pAcRP23 transfer vector without disrupting an internal *BamHI* site in the JHE gene. After transfection, this pAcRP23.JHE transfer vector places the JHE under the control of the polyhedrin promoter of the AcNPV^{13,14}. Thus, neither AcRP23.JHE nor the control virus expressing the HIV coat protein produced the polyhedron protein. Use of the polyhedron promoter reduces oral infectivity of the resulting virus.

fluoride ($I_{50} = 10^{-4}$ M) and strongly inhibited by a tri-fluoromethyl ketone, designed to be a selective JHE inhibitor [3-(1-decyl)thio-1,1,1-trifluoropropan-2-one; $I_{50} = 10^{-9}$ M] (ref. 4). In each case, the catalytic activity could be precipitated by a polyclonal antibody raised to the affinity-purified JHE of *H. virescens*⁷.

The natural haemolymph enzyme of *H. virescens* and the enzyme expressed by cells of *S. frugiperda* infected with AcRP23.JHE were both purified by affinity chromatography as described previously⁴. However, analysis by SDS-PAGE with western and glycan¹⁷ blotting indicated that the proteins were immunologically similar but had slightly different relative molecular masses as a result of extensive glycosylation of JHE in the *S. frugiperda* cells (Fig. 2). After the natural and expressed proteins were digested with endonuclease F to remove sugars, they had identical relative molecular masses. A major advantage of the baculovirus system is that it carries out many post-translational modifications, and insect JHEs from several species are known to have varying levels of glycosylation^{5,6}. However, the extensive glycosylation carried out by Sf21 cells in this study indicates that an investigation of post-translational modification in different host cells for engineered AcNPVs may be needed.

When crude supernatant from AcRP23.JHE-infected cells of *S. frugiperda*, or JHE purified by open-column and high-per-



formance liquid chromatography anion exchange chromatography, was injected into larvae of *Manduca sexta*, the caterpillars turned black at the next molt. This effect could be reversed by the application of epofenonane (a juvenoid lacking the methyl ester of JH; Fig. 3a)¹⁸. Blackening is a common bioassay for chemical anti-juvenile hormones^{19,20} and has been used to characterize the biological activity of natural enzymes after affinity purification²¹. This indicates that the expressed JHE does have anti-juvenile hormone activity.

When the polyhedrin minus AcRP23.JHE was fed to first instar larvae of *T. ni*, the feeding and growth of many of the infected larvae were profoundly reduced compared with either untreated control larvae or larvae treated with a control virus (Fig. 3b). The blood JHE levels in all of the stunted larvae assayed approached the levels normally seen at ecdysis, but were only about 10% of the levels seen in the normal last larval instar (Fig. 4a). On the basis of these data, and the observation that the level of JHE activity in the haemolymph rises in control animals between head capsule slippage and ecdysis²², we suggest that a reduction in JH titre caused by high JHE may lead to this reduction in feeding (Fig. 4b). When the infected larvae were treated with a juvenoid, feeding increased and growth was partially restored. These levels of juvenoid had no significant effect on the rate of growth in the early instars of control larvae or on larvae infected with similar doses of control virus (the

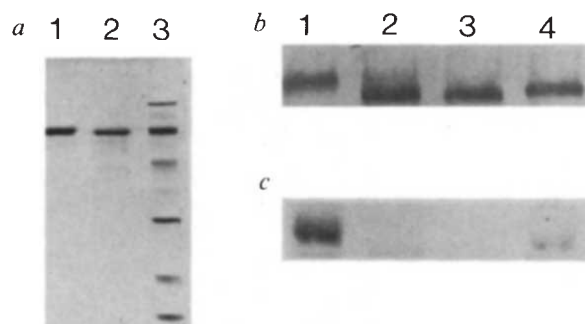


FIG. 2 Analysis of JHE purified by affinity chromatography from the haemolymph of fifth instar larvae of *H. virescens* and from the growth medium of cells of *S. frugiperda* infected with AcRP23.JHE. **a**, Sodium dodecyl sulphate polyacrylamide gel electrophoresis on a 12% gel stained with Coomassie Blue reveals slight differences in relative molecular mass (M_r). Lane 1, 2 µg of JHE expressed by the recombinant baculovirus (66K); lane 2, 2 µg of JHE from haemolymph (62K); lane 3, M_r standards of 97, 67, 43, 31, 22 and 14K. The data from a western blot (**b**) and a glycan blot (**c**) demonstrate that the difference in M_r is due to differential glycosylation of JHE. The central lanes show the effects of digestion with an endoglycosidase. **b**, **c**, Lanes 1, undigested JHE from recombinant baculovirus; lanes 2, digested JHE from recombinant baculovirus; lanes 3, digested JHE from haemolymph recombinant baculovirus; lanes 4, undigested JHE from haemolymph. In (**b**), the two digested preparations showed shifts to identical mobility. The immunologic similarity between the two purified preparations was revealed when the filter was probed with polyclonal antibodies developed against JHE from haemolymph⁷. In (**c**), the digested proteins lost their glycan-staining ability when they were treated according to the instructions of a glycan-staining kit¹⁷, showing that all oligosaccharides had been removed. **METHODS.** The two purified preparations were digested with an endoglycosidase selective for *N*-linked oligosaccharides (Peptide:N-acetylglucosidase F, Boehringer Mannheim Biochemicals) and analysed by immunoblotting (**b**) and blotting specifically for glycans (**c**) on proteins transferred to nitrocellulose filters by standard procedures. Gels **b** and **c** were run for 90 min rather than 60 min and the bands of interest were enlarged to emphasize differences in M_r .

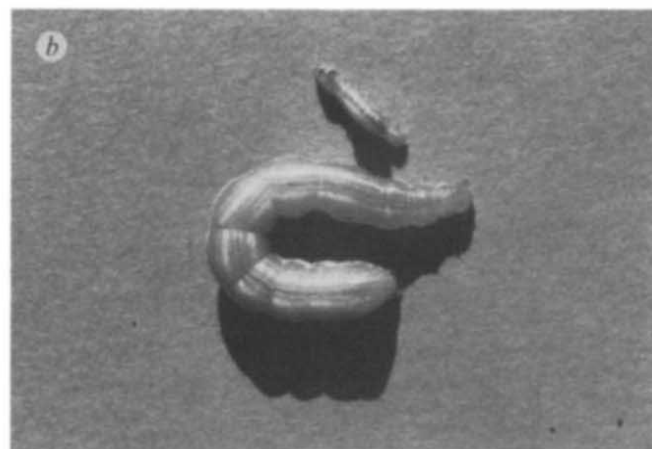
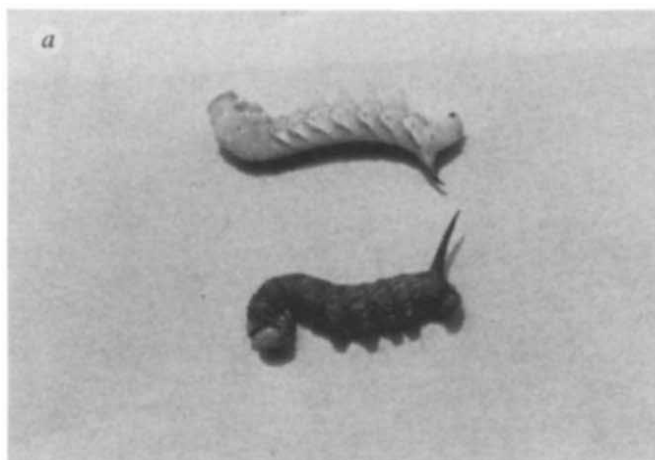


FIG. 3 The effects of expressed JHE on insects. *a*, The effects of JHE injected into mid-second instar larvae of *M. sexta*. *b*, The effects of infection of first instar larvae of *T. ni* with AcRP23.JHE.

METHODS. *a*, The top larva was injected with 5 μ l of a 1 mg ml⁻¹ solution of bovine serum albumin. The lower larva was injected with 50 pmol of purified JHE from AcRP23.JHE-infected *S. frugiperda* cells. The photograph was taken shortly after the molt to the third larval stadium, with the cuticular

blackening in the lower animal clearly demonstrating the anti-juvenile hormone effects of the expressed enzyme. *b*, The smaller larva was infected with the engineered virus and development was halted in the third larval stadium whereas the larger insect of the same age was not infected. Insects treated with the control virus expressing the HIV coat protein were slightly heavier than untreated controls.

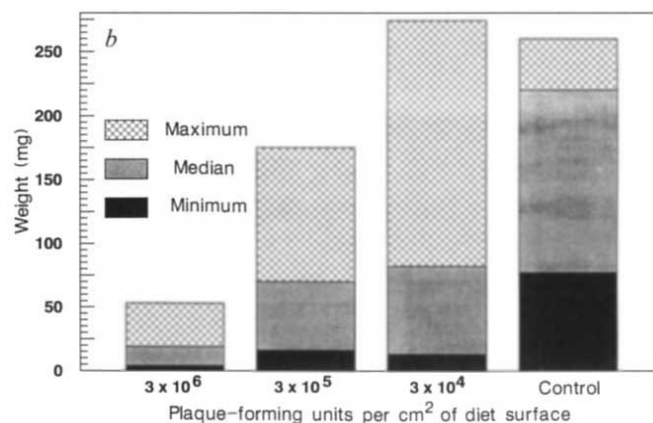
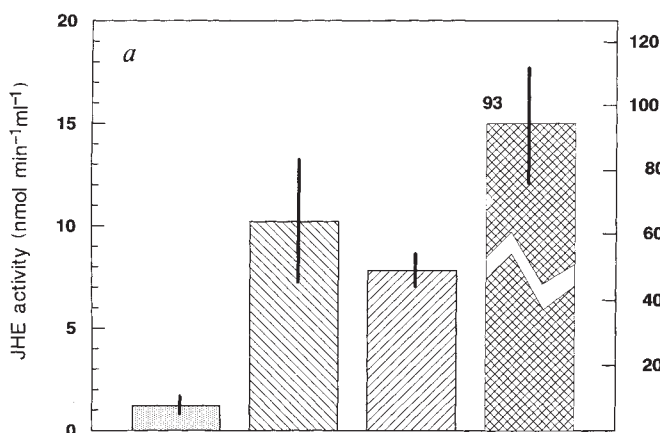


FIG. 4 The effects of AcRP23.JHE on larvae of *T. ni*. *a*, The effects of AcRP23.JHE infection on haemolymph JHE levels in *T. ni*. Following infection as described below, individual larvae were bled when the control larvae were in the third instar. The first bar indicates the low levels of JHE seen in the mid-third instar of feeding animals (or in larvae infected with wild-type AcNPV or AcNPV containing the gp120 gene²³). The second bar indicates the much higher levels of JHE found in infected larvae, whereas the third bar shows the JHE levels in 10 control insects at 'bubble head' (the time between head capsule slippage and ecdysis). For comparison, the fourth bar (note different scale on right) shows the level of JHE present at peak levels in the normal fifth instar (the average rate of hydrolysis is indicated above it). *b*, Minimum, maximum and median weights of larvae exposed to AcRP23.JHE as first stadium larvae. Median weights are shown because some insects are not infected at lower doses. The mean weights of animals

at the two higher concentrations are significantly different from the control population ($P=0.05$). Topical application of the juvenoid epofenonane (3 μ g given once in mid-first stadium and again in mid-second stadium) or addition of methoprene (200 μ g cm²) or epofenonane (20 μ g cm²) to the surface of the diet led to a significant increase in the weights of larvae treated with AcRP23.JHE whereas there was no significant effect on early instars of control larvae or larvae treated with AcNPV.gp120²³ ($P=0.05$).

METHODS. Larvae were treated by placing the virus on the surface of the diet of a 30 ml diet cup (7 cm² surface). An egg sheet containing approximately 10 eggs laid during a carefully timed period was placed in the lid of the cup and the cup inverted over the sheet. For this experiment, each dosage was repeated three times with 10 larvae per replicate. After 4 days at 24°, the larvae were transferred to individual containers and monitored daily for 6 days.

identical polyhedrin minus AcNPV coding for the secreted gp120 coat protein of the human immunodeficiency virus (HIV)²³. Larvae infected with this control virus were the same size or slightly larger than untreated control insects until they became moribund.

This reduction in feeding is an attractive aspect of this system, as one of the major problems involved with the use of baculoviruses for insect control is the feeding damage caused by infected insects²⁴. However, these effects were only seen after treatment of first instar larvae. There are several possible expla-

nations for this observation. In later stages, the low level of JHE produced under the control of a very late promoter is unable to overcome hormone biosynthesis. It also is likely that viral-induced production of an ecdysteroid UDP-glucosyl transferase reduces the effects of JHE²⁵. This gene is present in both AcRP23.JHE and the control virus expressing the HIV coat protein. As yet, it is not known why *in vivo* JHE expression is so much lower than *in vitro* expression. Despite its stability in the face of a variety of reagents, both the natural JHE of *M. sexta*²¹ and the JHE expressed from the vector are inactivated

rapidly *in vivo* (R. Ichinose, B. F. McGutchen and T.N.H., unpublished observations). If these problems of *in vivo* instability and low and late expression can be overcome, the JHE gene offers a very attractive tool for the development of genetically engineered insecticides and, at the very least, it will serve as a useful reporter gene in expression systems. □

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Binding of the *Drosophila* Sex-lethal gene product to the alternative splice site of transformer primary transcript

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SOMATIC sexual differentiation in *Drosophila melanogaster* is accomplished by a hierarchy of genes^{1–5} of which one, *Sex-lethal* (*Sxl*)^{6,7}, is required for the functional female-specific splicing of the transcripts of the immediately downstream regulatory gene, *transformer* (*tra*). The first exon of the *tra* primary transcript is spliced to one of two acceptor sites. Splicing to the upstream site yields a messenger RNA which is neither sex-specific nor functional, but that produced after splicing to the downstream acceptor site yields a functional female-specific mRNA. Here we address the question of how the *Sxl* gene product determines the alternative splicing of *tra* primary transcripts. One suggestion is that non-sex-specific splicing to the upstream acceptor is blocked in female flies by sex-specific factors⁸, but neither the identity of the female-specific factors nor the mechanism of the blockage has been specified. We have now performed co-transfection experiments in which *Sxl* complementary DNA and the *tra* gene are expressed in *Drosophila* Kc cells. Moreover, we find that female *Sxl*-encoded protein binds specifically to the *tra* transcript at or near the non-sex-specific acceptor site, implying that the female *Sxl* gene product is the *trans*-acting factor that regulates the alternative splicing.

When a plasmid expressing the *tra* gene under the control of the promoter of the copia-element long terminal repeat (LTR)¹³ (plasmid copia-*tra*) was transfected into Kc cells, the non-sex-

specific mRNA was exclusively generated (data not shown). To analyse whether *Sxl* gene products affect *tra* pre-mRNA splicing, we co-transfected the copia-*tra* plasmid with male- or female-specific *Sxl* cDNA located downstream of the *Drosophila* heat-shock-protein 70 gene (*HSP70*) promoter¹⁴ (plasmid hsp-*Sxl*). Only non-sex-specific splicing of *tra* transcripts was found when the copia-*tra* DNA was co-transfected either with the hsp vector or with the male-specific *Sxl* cDNA (hsp-*Sxl* M1) (Fig. 1b; lanes 1, 3). By contrast, female-specific splicing of *tra* transcripts was additionally observed on co-transfection of the copia-*tra* plasmid with the female-specific *Sxl* cDNA (hsp-*Sxl* F1) or with a mixture of hsp-*Sxl* M1 and hsp-*Sxl* F1 (Fig. 1b; lanes 2, 4). With hsp-*Sxl* F1, about 80% of *tra* RNAs were female-specific. That hsp-*Sxl* M1 did not interfere with the effect of hsp-*Sxl* F1 is consistent with earlier genetic analyses indicating that the product from male-specific *Sxl* transcripts is not functional^{1–3}. With a plasmid containing a frame-shift mutation in the coding region of female-specific *Sxl* cDNA, the amount of female-specific *tra* mRNA was drastically reduced (Fig. 1b; lane 5). The fraction of female-specific *tra* mRNA decreases in proportion to the amount of transfected hsp-*Sxl* F1 (data not shown), indicating that female-specific splicing of *tra* pre-mRNA depends on the dosage of the *Sxl* gene product. When a *transformer-2* (*tra-2*) cDNA^{15,16} was co-transfected with the copia-*tra* plasmid, no female-specific *tra* mRNA was detected (Fig. 1b; lane 6), consistent with *tra-2* not acting upstream of *tra*¹¹.

To clarify how the female-specific *Sxl* gene product controls alternative splicing, we constructed two plasmids containing a mutant *tra* gene in which either one of the two acceptor sites had been deleted (Fig. 2a). When the mutant plasmid containing a deletion of the non-sex-specific acceptor site (plasmid copia-*tra*ΔN; mutation *tra*ΔN) was co-transfected with hsp-*Sxl* M1 or F1, *tra*ΔN pre-mRNA was efficiently spliced at the female-specific acceptor site irrespective of the presence of the functional *Sxl* gene product (Fig. 2b; lanes 1, 2). By contrast, with the mutant in which the female-specific acceptor site had been deleted (plasmid copia-*tra*ΔF; mutation *tra*ΔF), *tra*ΔF pre-mRNA was spliced efficiently at the non-sex-specific acceptor site only in the absence of a functional female-specific *Sxl* gene product (Fig. 2b; lanes 3, 4). Essentially similar results were reported when mutant *tra* genes were introduced into either male or female flies⁸, but the product of *Sxl* was not shown to be directly responsible for the *tra* alternative splicing. Our results strongly indicate that the functional *Sxl* gene product acts at or near the non-sex-specific acceptor site to inhibit the non-sex-specific splicing of *tra* pre-mRNA.

If this interaction occurs, we would expect that the *Sxl* protein binds specifically to the transcript close to the non-sex-specific acceptor site. We tested the binding activity of the *Sxl* protein by north-western-blotting analysis of the female-specific *Sxl* gene product overproduced in *Escherichia coli* by the T7 promoter system¹⁷. All of the proteins from *E. coli* overproducing the *Sxl* protein were subjected to SDS-PAGE and then blotted onto nitrocellulose membrane. The blotted membrane was probed with various *tra*-derived RNAs synthesized *in vitro* (Fig. 3a). The *tra* RNA containing the non-sex-specific acceptor site bound strongly to a protein of relative molecular mass (*M_r*) 41,000 (Fig. 3b; lane 2), consistent with the size of the *Sxl* protein predicted to be encoded by the female-specific *Sxl* cDNA sequence. This protein was also detected by an anti-serum raised against the *Sxl* protein (data not shown). The *tra* RNA did not bind to any proteins from *E. coli* cells carrying the vector without the *Sxl* cDNA (Fig. 3b; lane 1). To determine which region of *tra* RNA binds to the *Sxl* protein, we tested *tra*ΔN and AF130, representing the deleted sequence of *tra*ΔN, RNA probes for binding activity (Fig. 3a). No binding was found with *tra*ΔN RNA, whereas AF130 RNA bound to the *Sxl* protein (Fig. 3b; lanes 3, 4). Binding of the protein to *tra* RNA was strongly inhibited by addition of poly(U), but not by poly(C) (Fig. 3b;

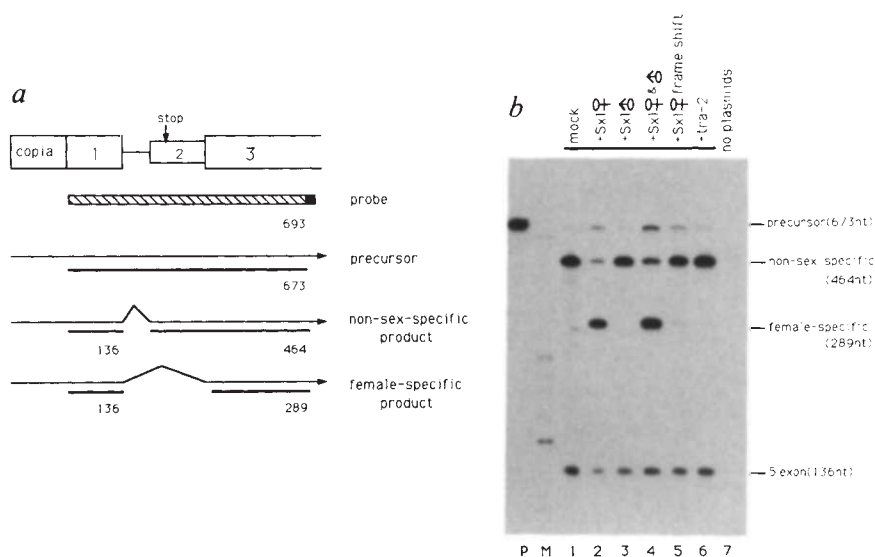
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lanes 7, 6). The Sxl protein did not bind to an RNA prepared from *fushi-tarazu* (*ftz*)¹⁸ containing two exons and one intron (Fig. 3b; lane 8). These results show that the Sxl protein specifically binds to a region of *tra* RNA which includes the non-sex-specific acceptor site, and indicate that this region contains U-rich sequences. Indeed, three U-to-C substitutions in the U tract located upstream of the non-sex-specific acceptor

site of *tra* pre-mRNA (Fig. 3a) abolish regulation of *tra* splicing in flies⁸. An RNA probe synthesized from *tra* containing these U-to-C substitutions⁸ (probe *traM*) did not bind to the Sxl protein (Fig. 3b; lane 5). When plasmid *copia-traM* is co-transfected with plasmid *hsp-Sxl F1*, only the non-sex-specific mRNA is detected, as is the case with *copia-traΔN* (data not shown).

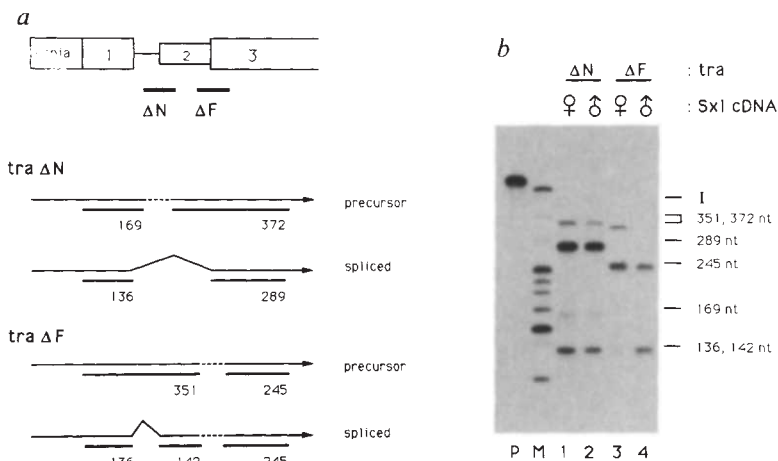
FIG. 1 Effect of *hsp-Sxl F1* cDNA products on the splicing patterns of *copia-tra* pre-mRNAs in Kc cells. **a**, Schematic representations of *copia-tra* pre-mRNA and expected products in RNase protection analysis. Top boxes and the line between the boxes represent the exon and the intron sequences, respectively. Transcription is initiated in the *copia*-element LTR sequences. The stop codons exist in-frame in the second exon. Probe RNA used in RNase protection assay is shown immediately below. The structures and sizes (in nucleotides, nt) of protected fragments corresponding to each RNA species are shown in the lower three lines. **b**, Co-expression analyses of *copia-tra* with *hsp-Sxl* constructs in Kc cells. RNA products were analysed by RNase protection assay using the *tra* antisense RNA shown in **a**. Either *hsp-Sxl F1* (Sxl ♂) or *hsp-Sxl M1* (Sxl ♀), or both, were individually transfected with *copia-tra* (lanes 2, 3, and 4, respectively). A frame-shift mutation (4-base pair (bp) insertion) was introduced into *hsp-Sxl F1* cDNA at the 41st codon, and the resulting plasmid, *hsp-Sxl F1 frame-shift*, was co-transfected (lane 5). The chloramphenicol acetyltransferase (CAT) gene coding region or the *tra-2* cDNA fused to the *HSP70* promoter was also co-transfected with *copia-tra* (lanes 1, 6). RNAs from untransfected Kc cells were also examined (lane 7). P and M, probe RNA and size markers (the *HpaII* digests of plasmid pBR322), respectively. ♀, Female cDNA; ♂, male cDNA.

METHODS. A promoterless *tra* genomic fragment derived from plasmid pbsB1neo (ref. 9) was fused to the downstream promoter of *copia* LTR (cDm2055)¹³. The *Sxl* female cDNA (F1)⁶, the male cDNA (M1)⁶, and the *tra-2* cDNA (C1.3)¹⁶ were individually fused to the promoter sequence of the *HSP70* gene¹⁴ having strong promoter activity even at normal temperature. These fragments were inserted into plasmid pBSOCAT which includes the CAT gene coding region and the simian virus 40 (SV40) poly(A) signal derived from plasmid pSV2CAT²⁰. The *hsp-Sxl F1* construct was



digested at the *AccIII* site, and the 3' recessive ends of the linear fragment were filled with Klenow fragment. This DNA fragment was then religated. The resulting plasmid contained the 4-bp insertion at the 41st codon of the *Sxl* female product (*hsp-Sxl F1 frame-shift*). Kc cells were cultured in M3BF medium with 2% fetal bovine serum at 25 °C. Calcium-phosphate transfections were performed essentially as previously described²¹. The *EcoRI*-*HincII* fragment of plasmid pbsB1neo was inserted into plasmid pSP64 (ref. 22) in the opposite direction to the SP6 promoter sequence, and antisense RNAs were synthesized *in vitro* using SP6 RNA polymerase in the presence of [α -³²P]GTP²³. Total cellular RNA (~30 µg) was hybridized with the labeled RNA (~20 fmol) at 45 °C. RNase A (10 µg ml⁻¹) and T1 (125 units ml⁻¹) digestion was performed at 30 °C for 30 min, and the digested products were electrophoresed on a 5% polyacrylamide gel containing 8 M urea.

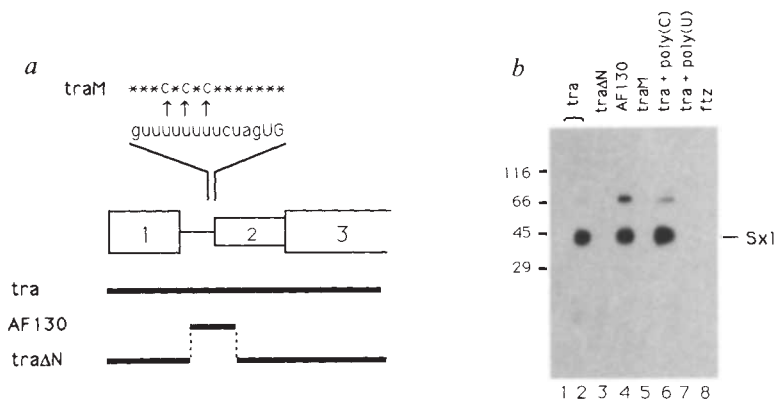
FIG. 2 The female-specific *Sxl* gene product inhibits the non-sex-specific splicing of *tra* pre-mRNA in Kc cells. **a**, Schematic diagrams of deletion mutants of *copia-tra* constructs (*copia-traΔN*, *copia-traΔF*), and the expected protection fragments from RNase protection assay. Mutation *traΔN* is a 132-base deletion of sequences including the non-sex-specific acceptor site of *tra* (corresponding to bases 151–276 of the sequence of Boggs *et al.*⁹). Mutation *traΔF* is a 77-base deletion of sequences including the female-specific acceptor site (corresponding to bases 327–403 in ref. 9). Top, boxes and the line between the boxes represent the exon and intron sequences, respectively; thick underlines indicate the deleted regions in *copia-tra*. The structures and sizes (in nucleotides, nt) of protected fragments corresponding to each RNA species are shown below. **b**, Co-expression analyses of the *copia-tra* deletion constructs with the *hsp-Sxl* constructs in Kc cells. The *copia-traΔN* (lanes 1, 2) and the *copia-traΔF* (lanes 3, 4) were individually co-transfected with *hsp-Sxl F1* (lanes 1, 3) or *hsp-Sxl M1* (lanes 2, 4). The nucleotide length of each band is shown on the right side of the panel. I, band corresponding to the endogenous non-sex-specific product (464 nucleotides, see Fig. 1). P and M, probe RNA and size markers (described in Fig. 1), respectively. **METHODS.** The *AlwNI*-*FspI* region in the *tra* sequence of the *copia-tra* construct was deleted, and the ends of the fragment were blunted with T4



DNA polymerase, then religated (*copia-traΔN*). The *EcoT22*-*EcoT14I* region was also deleted from *copia-tra* (*copia-traΔF*). Transfections and RNase-protection assay were performed as described in Fig. 1. RNA products were electrophoresed on a 5% polyacrylamide gel containing 8 M urea.

FIG. 3 The female-specific *Sxl* gene product overproduced in *E. coli* binds specifically to *tra* pre-mRNA at or near the non-sex-specific acceptor site. *a*, Schematic representation of probe RNAs used in north-western analyses. Boxes and the line between the boxes represent exon and intron sequences of *tra*, respectively. The regions covered by each RNA probe are shown by thick solid lines. Probe *traM* covers the same region as *tra* except that three of the eight consecutive U residues at the non-sex-specific acceptor site are substituted by C residues. Small and capital letters represent nucleotide sequences of the non-sex-specific intron and the following exon, respectively. *b*, Binding analyses of the female *Sxl* protein and *tra*-derived RNAs by north-western blotting. Total proteins from *E. coli* with (lanes 2–8) or without (lane 1) the female *Sxl* gene product were electrophoresed and blotted onto nitrocellulose membrane. The membrane was then incubated with RNA probes *tra* (lanes 1, 2, 5, 6), *traΔN* (lane 3), AF130 (lane 4), *traM* (lane 7) and *ftz* (lane 8). In two cases, poly(U) or poly(C) was added instead of yeast RNA as competitor in the binding reaction (lanes 5, 6). Positions of *M_r* markers are shown (*M_r* × 10⁻³ (left)).

METHODS. The *EcoRI* fragment of plasmid pHSB1neo was inserted into plasmid pSP65 (ref. 22) (pSPtra). The *AlwNI*-*FspI* region in the *tra* sequence of plasmid pSPtra was deleted to produce pSPtraΔN. The *AlwNI*-*FspI* blunted fragment was inserted into plasmid pSP73 at the *EcoRV* site (pSPAF130). The *Sall*-*BglII* fragment of the *ftz* genomic clone DmV61H3.5 (ref. 18) was inserted into plasmid pSP64 at the *Sall*-*BamHI* site (pSPftz). Oligonucleotide-directed mutagenesis using single-stranded *tra* DNA in M13 phage was performed essentially as described²⁴. Mutant *tra* DNA was recloned into plasmid pSP65 (pSPtraM). Plasmids pSPAF130 and pSPftz were digested with *EcoRI* and *XhoI*, respectively. Other plasmids were linearized with *HincII*.



RNA probes were synthesized *in vitro* using these linearized fragments as template in the presence of [α -³²P]GTP²³. The *BstBI*-*EcoRI* fragment including the entire coding region of female *Sxl* cDNA was inserted into the *BamHI* site of plasmid pET3 (ref. 17), the plasmid vector for selective expression of cloned DNA by T7 RNA polymerase. The plasmids were introduced into *E. coli* BL21(DE3)¹⁷. Total proteins were electrophoresed on a 15% SDS-polyacrylamide gel, and transferred onto nitrocellulose membrane. The binding reaction was performed under conditions containing 1–2 × 10⁴ c.p.m. RNA probe and 8 μg yeast transfer RNA (or either poly(U) or poly(C)) in 1 ml binding buffer (10 mM HEPES buffer, pH 7.9, 150 mM KCl, 1 mM EDTA, 0.25% skim milk, 0.05% (v/v) 2-mercaptoethanol). The membrane was washed several times in the binding buffer.

Several distinct male- and female-specific transcripts are produced from the *Sxl* locus¹⁹. We have shown here that the *Sxl* product from a single female-specific *Sxl* cDNA derived from one of these transcripts is sufficient to activate the female-specific splicing of *tra* transcripts. It remains to be found whether the products from other female-specific *Sxl* transcripts have the same function. We have also shown that the female-specific acceptor site of *tra* pre-mRNA is constitutively active in the absence of the non-sex-specific acceptor site, and that it is the use of the non-sex-specific acceptor site that is negatively controlled by the female-specific *Sxl* gene product. It is therefore probable that female-specific splicing *in vivo* results from the blocking of the dominant non-sex-specific splicing reaction by binding of the female-specific *Sxl* gene product to the non-sex-specific acceptor site. Our results therefore support the model of Sosnowski *et al.*⁸. This conclusion is now being more directly examined by analysing the *in vitro* splicing reaction. □

Transactivation of *c-fos* and β -actin genes by *raf* as a step in early response to transmembrane signals

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A PRIMARY response to many growth factor-induced transmembrane signals is the rapid activation of transcription of the proto-oncogene *c-fos* and other early-response genes, including the β -actin gene^{1,2}. The *c-raf* gene encodes a cytoplasmic serine/threonine kinase, *raf-1* (ref. 3), whose activity is also responsive to transmembrane signals^{4,5} and which in mutant form can transform cells⁶. Here we show that in transient assays, the *v-raf* protein, which is a constitutively activated oncogenic counterpart of *raf-1*, can transactivate transcription from two early-response promoters, including the *c-fos* promoter from human and murine cells and the human β -actin gene promoter. Multiple elements of the human *fos* promoter, including the dyad symmetry element necessary for growth-factor induction, an octanucleotide direct repeat element, and the region spanning the sequence from nucleotides -225 to -99 can all serve as targets for *raf* induction. The *c-myc* promoter and two adenovirus-2 early promoters are not induced. These findings indicate that *raf* kinase, when activated by a transmembrane signal or by mutation of a regulatory domain, can phosphorylate a factor(s) capable of regulating transcription of the *c-fos* and actin genes. The oncogenic form of *raf* may transform by constitutively activating early response proto-oncogenes such as *c-fos*.

To determine whether *v-raf*, the transforming gene of the

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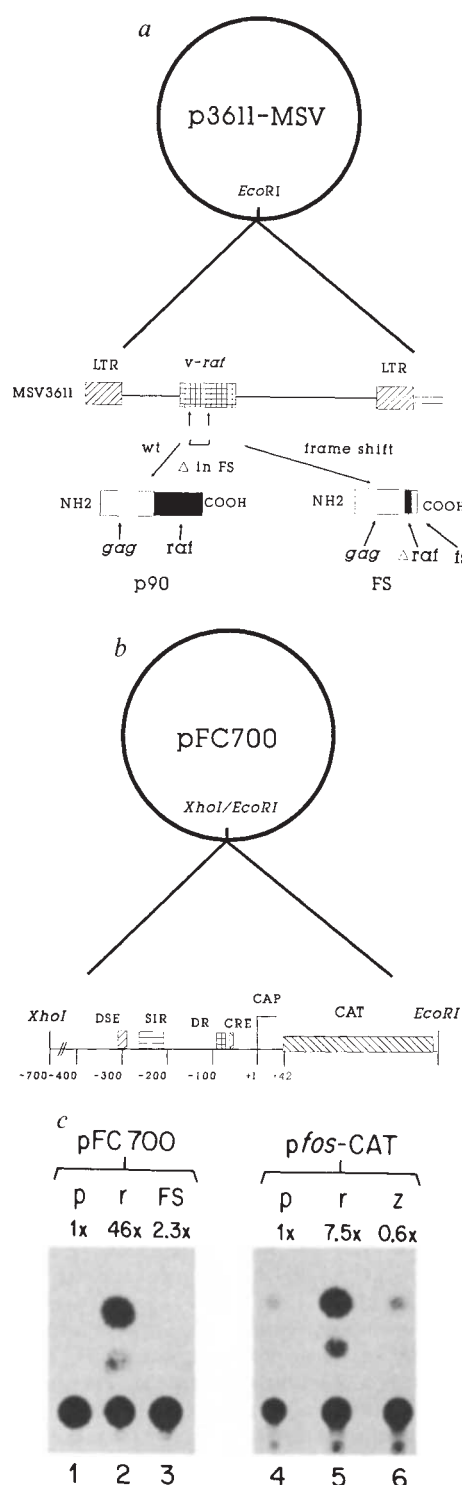


FIG. 1 Assay of *c-fos* promoter plasmids for transactivation by *raf* protein. **a**, Structure of plasmid p3611-MSV, a pBR322 clone of the 3611-MSV murine sarcomavirus provirus which encodes the *gag-raf* fusion protein, *v-raf*⁶. This plasmid was modified by deletion of a 444-base pair *Stu*I (New England Biolabs) fragment spanning positions 129 to 573 of the *v-raf* coding region. This yields plasmid *praf*FS, which has a stop codon at position 640 and expresses a frame-shifted and truncated product that retains viral *gag* sequences but lacks the *v-raf* kinase domain. **b**, Structure of plasmid pFC700 (ref. 10), in which 700 residues of the 5' flanking region of the human *c-fos* gene are cloned in pUC19 and are linked to the *CAT* surrogate gene. **c**, Human and murine *c-fos* promoters assayed for transactivation by *v-raf*. Plasmid pFC700 was co-transfected into NIH 3T3 cells with pBR322 (p; lane 1) or p3611-MSV (r; lane 2) or *praf*FS (FS; lane 3). Plasmid *pfos*-CAT (residues -356 to +109 of the murine *c-fos* gene 5' flanking region linked

to the *CAT* surrogate gene)¹¹ was similarly co-transfected with pBR322 (lane 4) or p3611-MSV (lane 5) or *pzipneo*¹² (z; lane 6). The cells were made quiescent by incubation in low serum and assayed for CAT activity. The increased induction of CAT relative to the pBR322 control is shown above each lane.

METHODS. Cells were passaged in DMEM plus 10% calf serum and plated at a density of 1×10^6 cells per 10 cm plate 24 h before transfection. Transfection was by calcium phosphate co-precipitation³⁴ using 10 μ g of CAT plasmid DNA plus 10 μ g of *v-raf* or control plasmid DNA per 10-cm dish. After 12–16 h, transfected cells were serum-starved by incubation in DMEM supplemented with 0.5% calf serum for 24 h. Cells were then collected, the protein extracted and assayed for CAT activity³⁵ by scintillation counting of the acetylated and unacetylated forms of [¹⁴C]chloramphenicol.

3611-MSV murine sarcoma virus⁶, is capable of activating transcription from the *c-fos* promoter, we co-transfected a plasmid clone of the provirus p3611-MSV (Fig. 1a), which encodes the *v-raf* kinase, into NIH 3T3 cells together with plasmids containing the *c-fos* promoter linked to a chloramphenicol acetyltransferase (*CAT*) surrogate gene. The *v-raf* protein differs from *raf*-1, its cellular counterpart, by deletion of an amino-terminal domain that negatively regulates *raf*-1 kinase activity and by substitution of viral *gag* sequences^{7–9}. This deletion renders the *v-raf* kinase constitutively active. We reasoned that if induction of the *raf* kinase was a step in activation of *c-fos* transcription by growth factors, then *v-raf* might induce *c-fos* even in the absence of a growth factor signal. Because *c-fos* transcription is transient and *c-fos* messenger RNA unstable, *c-fos* mRNA in transfected cells would decay and be an unreliable index of the response to *v-raf*. But the response of the *c-fos* promoter linked to a *CAT* surrogate gene could be monitored by assaying for stable *CAT* enzyme. We transfected 3T3 cells with the plasmid pFC700 (ref. 10) (Fig. 1b), in which the human *c-fos* promoter with 700 nucleotides of 5' flanking DNA is linked to the *CAT* surrogate gene or with *pFosCAT*¹¹, a murine *c-fos* promoter–*CAT* plasmid with 356 residues of 5' flanking sequence. Each plasmid was co-transfected with either p3611-MSV, or pBR322 as a control. The cells were then made quiescent by incubation in 0.5% serum for 24 h and assayed for *CAT* activity. As shown in Fig. 1c, lanes 1 and 2, *v-raf* stimulates the human *c-fos* promoter 46-fold relative to the pBR322 control, and the murine *c-fos* promoter, 7.5-fold (lanes 4 and 5). A frameshift mutant of p3611-MSV, *v-raf*FS (Fig. 1a), fails to induce *c-fos* transcription (Fig. 1c, lane 3) and in some cases represses basal *c-fos* promoter activity (data not shown). The plasmid *pzipneo*¹², which contains the long terminal repeat of p3611-MSV but no *raf* sequences, does not induce *pFosCAT* (Fig. 1, lane 6). Therefore the induction of *c-fos* transcription requires *v-raf* protein and not derepression by promoter competition as the mechanism of transactivation.

The ability of *v-raf* to replace serum as an inducer of *c-fos* suggested that a second serum-inducible early-response gene, the β -actin gene^{1,2}, might also be stimulated by *v-raf* and that promoters outside the early-response class might not be induced. Figure 2 shows that pH β -APr-1-CAT (human β -actin promoter plus first exon and intron, linked to *CAT*)¹³, is induced in quiescent cells 52-fold by p3611-MSV (lanes 1 and 2) and is slightly repressed by *praf*FS (lane 3). In contrast, pE2CAT and pE3CAT (plasmids with the E2 and E3 early promoters, respectively, of adenovirus-2 linked to *CAT*)^{14,15} which are inducible by the adenovirus-2 *E1a* gene, but not by serum, are not responsive to p3611-MSV (Fig. 2, lanes 4–7). We also looked at the *c-myc* promoter's response to *v-raf*, because *in vivo* *c-myc* responds to serum, but the kinetics are different from *c-fos* and *actin*^{1,16}. Interestingly, the *c-myc* promoter in plasmid *pmycpvpv* (human *c-myc* promoter linked to *CAT*) does not respond to *v-raf* either (Fig. 2, lanes 8 and 9). We conclude from these results that a second early-response promoter, that of the β -actin gene, can be induced by *v-raf* but two promoters induced by the viral transactivator *E1a*, are not responsive. The unrespon-

siveness of the *c-myc* promoter to *v-raf* and its distinct kinetics of induction by serum *in vivo*^{1,16} suggest that it is controlled by a mechanism different from that of *c-fos* and *actin*.

Several functional elements of the human *c-fos* promoter have been identified. These include the dyad symmetry element (DSE)¹⁷, the serum inducible repeat (SIR)¹⁸, an octanucleotide direct repeat (DR)¹⁹, a cyclic AMP response element (CRE)^{19,20} and a TATA box. To determine which regions of the *c-fos* promoter are required for the response to *v-raf*, we co-transfected *v-raf* with a series of plasmids with varying deletions of *c-fos* 5' flanking sequences (Fig. 3a). The results of several experiments are summarized in Fig. 3a and a typical experiment is shown in Fig. 3b. As before, *v-raf* strongly stimulates pFC700. Surprisingly, plasmid pFC225 (promoter truncation to -225) was even more responsive to *v-raf* than pFC700, although it has no DSE, an element required for serum stimulation, which we will show is a *v-raf* target. In contrast, plasmid pFC99 (truncation to -99) is not responsive, indicating that the critical residues must be in the -225 to -99 region. Plasmid pFC255/99, which lacks the -225 to -99 region but retains the residues between -700 and -225, including the DSE, gives a moderate response to *v-raf*. This indicated that the sequence upstream from -225 can partially respond to *v-raf* even when residues -225 and -99 are absent. pFC94/73, which lacks the DR elements, is significantly less induced than the wild type. pFC63/53, which has no CRE but retains all other elements, was induced to nearly the same extent as the wild-type plasmid, pFC700. Taken together, these results indicate that at least two regions of the *c-fos* promoter contribute to the *v-raf* response: one lies upstream between residues -700 and -225 and the other between -225 and -99; also, the CRE is not necessary for *v-raf* induction, and the DR sequence is required for full induction. Fisch *et al.*¹⁰ have reported that the DR element contributes to the basal level activity of the *fos* promoter.

To map the *v-raf* responsive sequences of the *fos* promoter, more precisely, we linked isolated elements to minimal unresponsive promoters and assayed them for inducibility. In Fig. 4a (lanes 1 and 2), it can be seen that the promoter region -700 to -225, which spans the DSE, is induced 42-fold by *v-raf* when linked to position -53 of the *fos* promoter (average 17-fold in

3 trials). The parent plasmid, pFC53 is induced only 2-fold (lanes 5 and 6) (no signal in two other trials). Lanes 3 and 4 show that pDSE-CAT (Fig. 4b), in which a minimal DSE oligonucleotide is linked to position -53, is induced 16-fold (average 6-fold in 4 trials). The decreased induction relative to the -700 to -225 construct could reflect the presence of other responsive sites in the -700 to -225 region that may synergize with the DSE, including a *raf*-responsive AP-1 site²¹. Lanes 7 and 8 show that the region spanning -225 to -99 confers inducibility of 9.6-fold (average 9-fold over two trials). This region is responsive to cyclic AMP²⁰, but because *raf*-1 is not activated by cAMP⁴, it is likely that cAMP and *raf* have separate targets. A construct in which three copies of an oligomer spanning the DR region of the human *c-fos* promoter are linked to a minimal thymidine kinase (TK) promoter (Fig. 4b) was induced 48-fold by *v-raf* (33-fold over 4 trials) (lanes 11 and 12). Constructs containing only one copy of this oligomer are not inducible, however, indicating that the DR on its own is probably inadequate (data not shown).

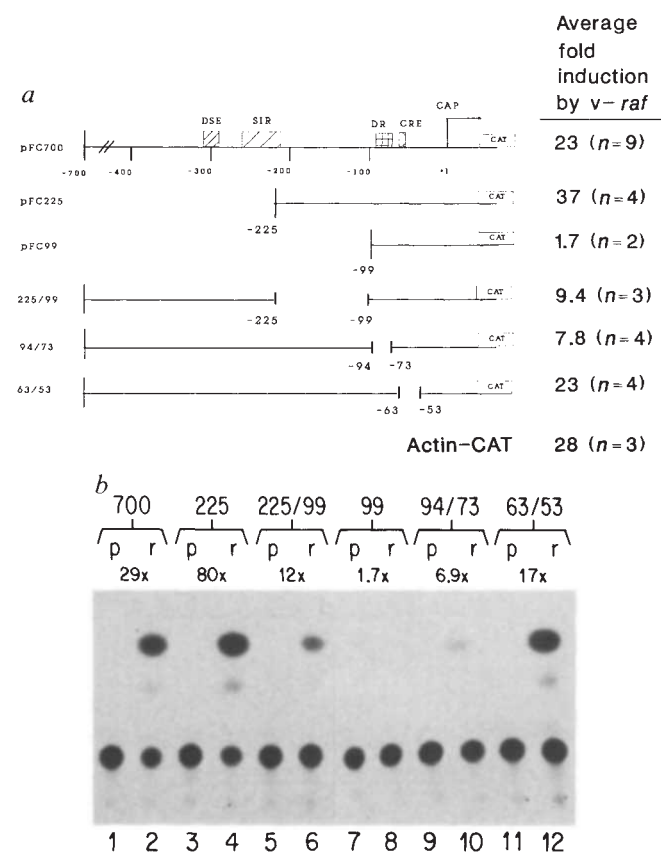


FIG. 3 Assay of regulatory elements of the *c-fos* promoter for response to *v-raf*. **a**, DNA sequences in the pFC series of human *c-fos* promoter-CAT surrogate gene plasmids. The structure of the parent of this series, pFC700 (see Fig. 1a) is compared with DNA elements in the human *c-fos* gene 5' flanking region which contribute to promoter activity and serum regulation. These elements include the dyad symmetry element (DSE)¹⁷, a serum-inducible region with repeat elements (SIR)¹⁸, a tandemly repeated octanucleotide sequence (direct repeat, DR)¹⁹, a cyclic AMP-response element (CRE)^{19,20}, the TATA box (TATA) and the 5' cap site. Modification of pFC700 gave a series of plasmids (gift from R. Prywes, T. Fisch and R. Roeder) lacking specific regulatory elements of the human *c-fos* promoter¹⁰ as shown. The fold induction of CAT enzyme activity encoded by these plasmids when co-transfected with p3611-MSV relative to co-transfection with control pBR322, has been averaged for several experiments and is given with the number of trials in parentheses. **b**, Plasmids pFC700, -225, -225/99, -99, -94/73 and -63/53 (ref. 10) (structures given in **a**) were cotransfected into NIH 3T3 cells with either pBR322 (p) or with p3611-MSV (r) and the cells assayed for CAT activity as described in the legend to Fig. 1. The fold induction by *v-raf* relative to the pBR322 control is shown.

FIG. 2 Specificity of transactivation by *v-raf* for promoters of early-response genes. Cellular and viral promoters were assayed for their response to *v-raf*. NIH 3T3 cells were co-transfected with plasmid p β -APr-1-CAT, a β -actin promoter-CAT surrogate gene plasmid¹³, plus either pBR322 (p; lane 1) or p3611-MSV (r; lane 2) or *praf*FS (FS; lane 3). The fold induction relative to the pBR322 control is shown above each lane. Adenovirus-2 early promoters (AdE2 and AdE3) were assayed for induction by *v-raf*. Cells were co-transfected with pE2CAT¹⁴ plus either pBR322 (lane 4) or p3611-MSV (lane 5), or with pE3CAT¹⁵ plus either pBR322 (lane 6) or p3611-MSV (lane 7). To test the response of the human *c-myc* promoter to *v-raf*, cells were co-transfected with the plasmid *pmyc*pv (gift from R. Dalla Favera; human *c-myc* genomic DNA extending from the *Pvu*II site 350 nucleotides upstream from the *P*₁ promoter, to the *Pvu*II site 522 residues downstream, linked to CAT) plus either pBR322 (lane 8) or p3611-MSV (lane 9). Transfections and assay of CAT enzyme activity were all as described in the legend to Fig. 1.

Our results show that an activated form of *raf* protein can stimulate *c-fos* and β -actin gene transcription, and so provide direct evidence linking *raf* to the activation of two early response genes. The presence of multiple elements within the *c-fos* promoter that are responsive to *raf* suggests that multiple factors may be influenced by the *raf*-1 kinase. One responsive element, the DSE, is required for transcriptional induction by growth factors and has also been shown by Kaibuchi *et al.*²² to be *raf*-inducible. The β -actin gene contains an element, the CARG box²³, which is homologous to the DSE and is a potential target for β -actin gene induction by *v-raf*. Both the DSE and the CARG box bind SRF, a nuclear factor that participates in the serum induction mechanism^{24,25} and requires phosphorylation for its interaction with the DSE²⁶. Although it has been reported that SRF is phosphorylated after stimulation of A431 cells by epidermal growth factor (EGF)²⁷, a regulatory role for such phosphorylation remains to be established. The DR, shown here to be a second *v-raf* responsive element, provides basal level activity in HeLa and A431 cells and binds a factor in HeLa extracts (S.J. and E.Z., unpublished observations). Little is known about the factors that associate with another *v-raf* responsive region lying between -225 and -99. The fact that pFC225, which contains this region and is responsive to *v-raf*, is not induced by serum¹⁰ suggests that *v-raf* may have targets in the *c-fos* promoter that are not regulated by serum. The increased induction of pFC225 over pFC700 is consistent with there being a negative regulatory element of the *fos* promoter

in the vicinity of the DSE. In establishing the mechanism of activation, it will be important to demonstrate that *v-raf* substrates correspond to those of *raf*-1.

After stimulation of NIH 3T3 cells by platelet-derived (PDGF), acidic fibroblast or epidermal growth factors or phorbol esters, *raf*-1 protein is rapidly phosphorylated and *raf*-1 kinase activity is elevated⁴. Also, *raf*-1 protein binds to the β -PDGF receptor after PDGF induces receptor tyrosine phosphorylation⁵. A variety of plasma membrane-generated signals, such as those from growth factor receptors or protein kinase C, may converge on *raf*-1 and induce its kinase activity⁵. *Raf*-1 in turn may relay the signal to other compartments of the cell such as the nucleus. *Raf*-1 also induces genes under the control of phorbol ester response elements, which bind the murine counterpart of the *fos-jun*-encoded complex²⁸. When considered with our results, this activation could reflect an induction of the synthesis of *fos* and *jun* protein. The pathway of early-response gene activation is notable in so far as several agents that function on this pathway have oncogenic counterparts: namely, PDGF, *v-sis*²⁹, EGF receptor, *v-erbB*³⁰; *c-fos* and *v-fos*³¹. We have added another oncogene product, *v-raf*^{6,32}, to this list.

No physiological substrate for *raf*-1 has yet been described and the relevant substrate(s) may be either cytoplasmic or nuclear. The binding of *raf*-1 to the PDGF receptor⁵ raises the possibility that the target for *raf*-1 may reside in the cytoplasm. The cytoplasmic dissociation of the transcription factor NF κ B from a repressor provides a precedent for a cytoplasmic mechanism for transcription factor activation³³. We are at present attempting to identify *raf*-1 substrates and to relate their phosphorylation to the activation of the DSE and the DR elements to deduce the role of *raf*-1 in early response gene induction and transmission of growth factor signals from the cytoplasm to the nucleus. □

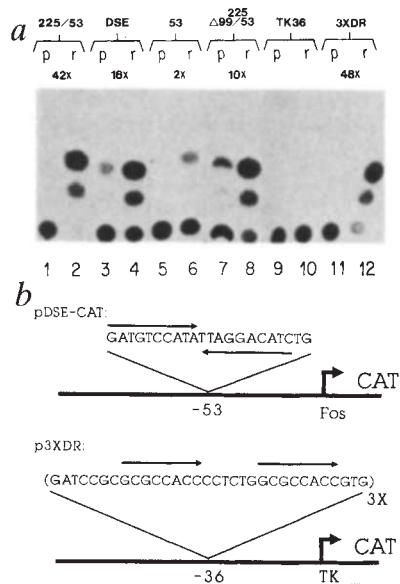


FIG. 4 Identification of specific *raf*-responsive elements in the human *c-fos* promoter. *a*, Assays for responsiveness to *raf* of isolated elements of the human *c-fos* promoter. These elements contained residues -700 to -225 (lanes 1 and 2), residues -319 to -297 (corresponding to the dyad symmetry element, DSE; see *b*) (lanes 3 and 4), and the region spanning -225 to -99 (lanes 7 and 8); they were all analysed in the same parent plasmid, pFC53, the -53 minimal human *c-fos* promoter. Analysis of pFC53 is shown in lanes 5 and 6. The direct repeat element is analysed using plasmid p3XDR (see *b*) and the corresponding parent plasmid, pTK36 (lanes 9 and 10). Transfection and assay were as described in the legend to Fig. 1. *b*, Structures of the DSE element inserted in pFC53 to yield pDSE-CAT (plasmid pFC53E of ref. 10) and the triplicated direct repeat oligonucleotide inserted in pTK36 (a truncation of the herpesvirus thymidine kinase (HSV-TK) promoter to residues -36) to yield p3XDR. p3XDR was constructed by annealing the complementary strands of the indicated 32-mer and cloning into the *Bam*H1 site of pTK36. The number of inserts was determined by restriction analysis but their orientation has not been established. Plasmid pTK36 was constructed by removing the *Bam*H1-*Bgl*II HSV promoter insert from pBLCAT2 (ref. 36) and replacing it with a *Bam*H1-*Bgl*II fragment of HSV-TK linker scanning mutant LS-47/-37 (ref. 37) which spans residues -36 to +51 of the HSV-TK promoter.

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Selection *in vitro* of an RNA enzyme that specifically cleaves single-stranded DNA

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THE discovery of RNA enzymes^{1,2} has, for the first time, provided a single molecule that has both genetic and catalytic properties. We have devised techniques for the mutation, selection and amplification of catalytic RNA, all of which can be performed rapidly *in vitro*³. Here we describe how these techniques can be integrated and performed repeatedly within a single reaction vessel. This allows evolution experiments to be carried out in response to artificially imposed selection constraints. We worked with the *Tetrahymena* ribozyme, a self-splicing group I intron derived from the large ribosomal RNA precursor of *Tetrahymena thermophila* that catalyses sequence-specific phosphoester transfer reactions involving RNA substrates^{4,5}. It consists of 413 nucleotides, and assumes a well-defined secondary and tertiary structure responsible for its catalytic activity. We selected for variant forms of the enzyme that could best react with a DNA substrate. This led to the recovery of a mutant form of the enzyme that cleaves DNA more efficiently than the wild-type enzyme. The selected molecule represents the discovery of the first RNA enzyme known to cleave single-stranded DNA specifically.

A secondary-structure model of the *Tetrahymena* ribozyme has been developed based on phylogenetic comparison with other group I introns⁶⁻⁸. Guided by this model, we prepared several structural variants of the ribozyme that lack one or more of the stem-loop structures of the wild-type (A. A. Beaudry and G.F.J., unpublished results). The ensemble of deletion mutants contained several variants that could cleave a DNA substrate. We used a subset of this ensemble to demonstrate that we could selectively amplify individuals best able to perform a chosen catalytic task, in this case transesterification with DNA.

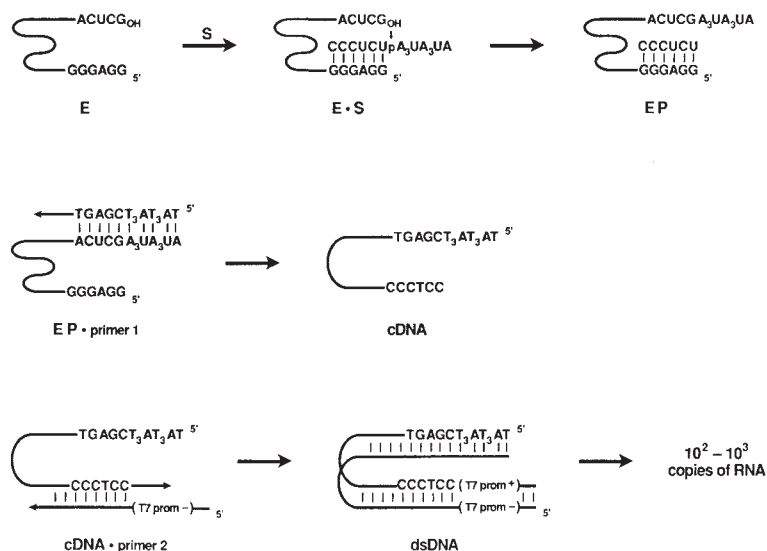
We assayed catalytic activity by performing an intermolecular addition reaction involving attack by the 3'-terminal guanosine

of the ribozyme at a phosphodiester bond after a sequence of pyrimidines located within a nucleic acid substrate³. The product of the reaction is the ribozyme joined to the substrate sequence that lies downstream from the target phosphodiester (Fig. 1, top). The format of the reaction was the same regardless of whether an RNA or DNA substrate was used. Selection occurs when an oligodeoxynucleotide primer is hybridized across the ligation junction and used to initiate the synthesis of complementary DNA (Fig. 1, middle). The primer does not bind to unreacted starting materials ($<10^{-6}$ compared with binding of reaction products) and therefore leads to selective reverse transcription of catalytically active RNAs. To amplify the selected materials, a promoter of T7 RNA polymerase is attached to the 3' end of the cDNA and the DNA is transcribed back to RNA^{9,10} (Fig. 1, bottom). Amplification occurs at the level of transcription owing to the high turnover of T7 RNA polymerase¹¹. Mutations can be introduced by replacing a portion of the cDNA with one or more mutagenic oligodeoxynucleotides and transcribing the partially mismatched template directly¹².

To demonstrate the ability of this reaction system to select RNAs that have novel catalytic function, we studied the cleavage-ligation reaction involving the *Tetrahymena* ribozyme and a DNA substrate. Although the ribozyme cleaves oligopyrimidine-containing RNA substrates¹³ and the chimaeric substrate dC₅rC (ref. 14), it was thought to be incapable of binding DNA productively to the active site¹⁴. Working with an ensemble of wild-type and mutant forms of the *Tetrahymena* ribozyme, we performed a series of reactions using either the RNA substrate GGCCUCU·A₃UA₃UA, the uracil-containing DNA substrate d(GGCCCTCU·A₃TA₃TA), or the DNA substrate d(GGCCCTCT·A₃TA₃TA). Surprisingly, several structural variants, including the wild-type, could cleave all three substrates.

Focusing on the DNA substrate d(GGCCCTCT·A₃TA₃TA), we performed selective amplification to recover the most reactive individuals from the assembled population of structural variants (Fig. 2). The ΔP9 single-deletion mutant proved to be the most efficient catalyst. This molecule contains a 93-nucleotide deletion at a position seven nucleotides from the 3'-terminus. After the first round of amplification (RNA' in Fig. 2) the selected RNAs still had the tag sequence A₃UA₃UA attached to their 3' end. The tag sequence is lost from about half of the material because of an RNA-catalysed site-specific hydrolysis reaction that occurs under the conditions of the transcription reaction¹⁵. After the

FIG. 1 Selective amplification of the *Tetrahymena* ribozyme (E) based on its ability to react with an oligonucleotide substrate (S). Top, L-21 form of the ribozyme binds an oligopyrimidine-containing RNA substrate by complementary pairing. The 3'-terminal G_{OH} of the ribozyme attacks the phosphodiester bond at a position after a sequence of pyrimidines, resulting in transfer of the 3' portion of the substrate to the 3' end of the ribozyme. Middle, product of the RNA-catalysed reaction offers a unique site for hybridization of an oligodeoxynucleotide used to initiate cDNA synthesis. Bottom, primer containing the T7 promoter is hybridized to the cDNA, the second strand of the promoter is completed, and the DNA is transcribed to RNA. Reaction conditions for transesterification: 1 μM ribozyme, 2 μM substrate, 30 mM EPPS, (pH 7.5) (N-2-hydroxyethyl-piperazine-N-2-propane sulphonate), 50 mM MgCl₂, 2 mM spermidine, incubated at 50 °C for 1 h. Reaction conditions for selective amplification: 20-fold excess of d(TAT₃AT₃CGAGT) primer was hybridized to the RNA by incubating at 65 °C for 5 min in the presence of 50 mM Tris buffer (pH 7.5) and 5 mM DTT, and then cooling rapidly to 0 °C. MgCl₂ (6 mM), 100 μM (each) dNTPs and 1 U μl⁻¹ AMV reverse transcriptase were added and the mixture was incubated at 37 °C for 20 min. RNA was destroyed by alkaline hydrolysis and monomers were removed by ethanol precipitation. A 20-fold excess of d(ATCGATAATACGACTCAC-TATAGGAGGAAAGTTATCAGGC) primer was hybridized to the cDNA as described above. MgCl₂, (15 mM), 2 mM spermidine, 100 μM (each) dNTPs, 2 mM (each) NTPs, 1 U μl⁻¹ AMV reverse transcriptase, 0.2 U μl⁻¹ DNA



polymerase I (Klenow fragment), and 20 U μl⁻¹ T7 RNA polymerase were added and the mixture was incubated at 37 °C for 1 h. Abbreviation: prom, promoter.

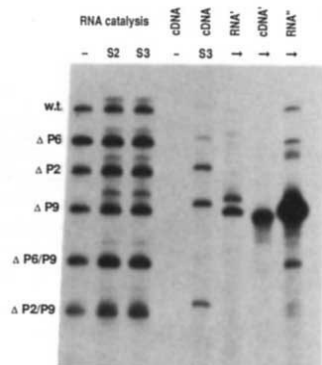


FIG. 2 Selective amplification of an ensemble of structural variants of the *Tetrahymena* ribozyme based on their ability to perform a cleavage-ligation reaction with a DNA substrate. Lanes 1–3, reaction with no substrate, the substrate d(GGCCCTCU·A₃TA₃TA) (S2), or the substrate d(GGCCCTCT·A₃TA₃TA) (S3), respectively. Lanes 4–5, selective cDNA synthesis of reaction products from lanes 1 and 3, respectively. Lanes 6–8, successive rounds of transcription and reverse transcription leading to amplification of selected materials. For reaction conditions, see legend to Fig. 1. Second round of cDNA synthesis used equimolar mix of d(CGAGTACTCCAAAC) and d(CGAGTACTCCGAC) primers to restore the 3' terminus of RNA. Deletion mutants are labelled according to the standard nomenclature for group I introns²³. RNAs were uniformly labelled with 1 μ Ci nmol⁻¹ [α -³²P] GTP; DNAs with 20 μ Ci nmol⁻¹ [α -³²P] dATP. A 1:50 dilution was made after each transcription, before gel loading. Materials were separated by electrophoresis in a 5% polyacrylamide–8M urea gel, an autoradiogram of which is shown. Abbreviation: wild type, w.t.

second round of amplification (RNA' in Fig. 2) the 3'-terminal G_{OH} is restored to all of the selected material. At this point the selected RNA could be used to begin another round of selective amplification, targeting either the same or a different nucleic acid substrate.

The selected Δ P9 mutant was isolated by PAGE and purified by chromatography on Sephadex G-50. Its sequence was confirmed by primer-extension analysis using reverse transcriptase in the presence of dideoxynucleotides¹⁶. The accuracy of RNA-catalysed DNA cleavage was determined by performing the reaction using a 5' ³²P-labelled DNA substrate. The substrate was sequenced by the chemical method¹⁷. Cleaved and uncleaved substrate were compared by alignment against a

ladder obtained by partial P1 nuclease digestion of the 5' [³²P]-labelled substrate. When the substrate d(GGCCCTCT·A₃TA₃TA) was used, the only product was an 8-mer having the sequence d(GGCCCTCT). Similarly, when the substrate d(CCCTCT·A₃TA₃TA₃) or d(CCCTCT·A₁₅) was used, the sole product was the 6-mer d(CCCTCT) (data not shown).

We compared the activity of the wild-type and Δ P9 mutant ribozyme in a cleavage-ligation reaction involving either the RNA substrate GGCCUCU·A₃UA₃UA or the DNA substrate d(GGCCCTCT·A₃TA₃TA) (Fig. 3). The Δ P9 mutant is more efficient than the wild-type in cleaving both substrates, and cleaves DNA more efficiently than the wild-type cleaves RNA. Both forms of the enzyme cleave RNA more efficiently than they cleave DNA, especially at early reaction times. The reaction with the uracil-containing DNA substrate d(GGCCCTCU·A₃TA₃TA) does not differ significantly from the reaction with standard DNA (data not shown).

Cleavage of DNA seems to be mechanistically similar to cleavage of RNA because deletions made outside the catalytic core of the ribozyme can result in enhanced DNA-cleavage activity. Oligopyrimidine-containing RNA substrates bind to the ribozyme by complementary pairing to an oligopurine-containing 'internal guide sequence'^{18,19}. Because both the RNA·RNA duplex and the RNA·DNA heteroduplex tend to exist in the helical A form^{20,21}, it is not surprising that an oligopyrimidine-containing DNA substrate can also bind to the internal guide sequence. Apparently, the detailed stereochemical differences between DNA and RNA binding are more than compensated by the effects of the Δ P9 deletion.

The transesterification reaction involving the *Tetrahymena* ribozyme and a DNA substrate results in joining of the ribozyme to the sequence of DNA that lies downstream of the cleavage site. This indicates that a self-splicing RNA could insert itself at specific locations in a DNA genome, in the same way that the *Tetrahymena* ribozyme inserts itself into a target RNA²². Site-specific hydrolysis at the phosphodiester bond after the 3'-terminal guanosine of the ribozyme¹⁵ would release the attached portion of the DNA substrate, freeing the ribozyme to perform another cleavage-ligation reaction.

We present this simple example of *in vitro* mutagenesis and selective amplification of an RNA enzyme to demonstrate the feasibility of controlled evolution experiments for the development of RNAs with novel catalytic function. Beginning with the Δ P9 mutant, we have now scattered random point mutations throughout the catalytic core of the ribozyme to produce a more complex population of variants. We will use this population to search for other related phenotypes in an attempt to further expand the catalytic repertoire of RNA. □

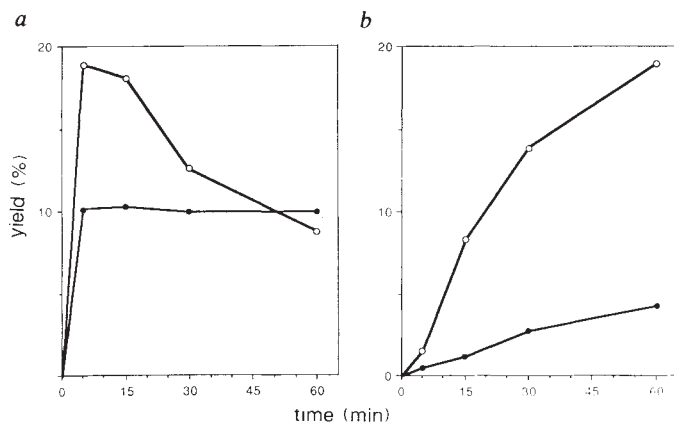


FIG. 3 Time course of the cleavage-ligation reaction involving the wild-type (●) and Δ P9 mutant form (○) of the *Tetrahymena* ribozyme using either the RNA substrate GGCCUCU·A₃UA₃UA (S1) (a) or the DNA substrate d(GGCCCTCT·A₃TA₃TA) (S3) (b). Reaction conditions: 20 nM ribozyme uniformly labelled with 1 μ Ci nmol⁻¹ [α -³²P] GTP, 2 μ M substrate, 30 mM EPPS (pH 7.5), 50 mM MgCl₂, and 2 mM spermidine, incubated at 50 °C in 7.5 μ l. Individual reaction mixtures were quenched at the indicated times by addition of 4 M urea and 50 mM sodium-EDTA. Reaction products were separated by electrophoresis in a 5% polyacrylamide–8M urea gel, cut from the gel, and quantitated by Cerenkov-counting using a liquid-scintillation counter.

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Antibodies produced in plants

A. Hiatt

Transgenic plant systems for the expression of mammalian antibodies offer opportunities for the study of plant metabolism and development. Agricultural production could provide virtually unlimited quantities of any antibody.

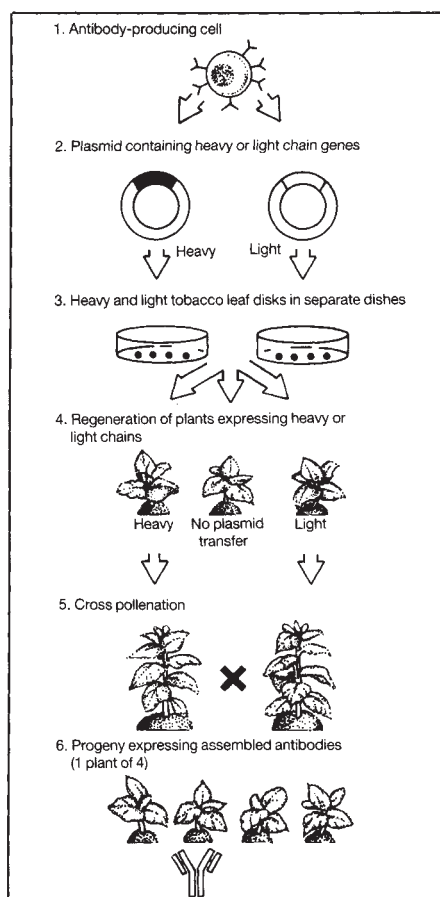
HETEROLOGOUS systems for the expression of mammalian antibodies will undoubtedly contribute a great deal towards our ability to isolate and manipulate immunoglobulins¹. The latest heterologous host system for antibody synthesis is plants. Techniques to generate transgenic plants have been perfected to the point where a foreign protein can be targeted to an organ of choice as well as to subcellular compartments.

Production of antibodies by plant cells offers a variety of new possibilities for basic research in plant biology as well as for large-scale production of antibodies for use as therapeutic, diagnostic or affinity reagents. The unparalleled capacity and flexibility of agricultural production suggests that antibodies derived from plants may be significantly less expensive than antibodies from any other source. Moreover, antibodies in plants may become useful reagents for manipulating agronomic traits and possibly for ameliorating symptoms of pathogenic infections, as well as for isolating and processing environmental contaminants or industrial by-products.

Plant transformation

Successful expression of an antibody in tobacco has recently been reported². A catalytic antibody³ was chosen to test the ability of the tobacco cell to assemble and process immunoglobulin chains without compromising functionality. cDNAs encoding heavy and light chains were first inserted into *Agrobacterium tumefaciens*, a soil bacterium that has proven to be very useful for transforming many types of plant cells⁴. The *Agrobacterium* is responsible for transferring the DNA into the plant cell where it is subsequently integrated into the genome. Transformed plant cells are then regenerated to become mature plants⁵.

The strategy used for antibody production was to transform tobacco leaf discs and regenerate separate plants expressing either the light or heavy chains (see figure). These plants were then sexually crossed to produce progeny-expressing functional antibody. Although the levels of expression varied widely, greater than one per cent of total protein constituted functional antibody in some plants. There is reason to believe that this level of expression can be augmented by using promoter elements capable of higher levels of transcription⁶. The antibody can easily be



Production of antibodies in tobacco plants. Primary regenerants transformed with *Agrobacterium* containing heavy or light chain cDNAs are sexually crossed to enable assembly of a functional antibody in resulting progeny.

purified from homogenized leaves in one affinity purification step. The catalytic properties of the tobacco-produced antibody allow a precise evaluation of kinetic parameters such as K_m , K_i and K_{cat} ; by these functional criteria, it is identical to the same antibody derived from hybridoma cells. Further characterization (for example, site of synthesis, secretion, glycosylation) will be reported elsewhere.

Of critical importance, is an evaluation of the immunogenicity of plant-derived antibodies in mammals. As plants do not contain sialyl transferase activity⁷, the terminal residues of the carbohydrate on the heavy chain will be different from mammals. In all probability, they will consist of xylose, fucose, and/or *N*-acetylglucosamine⁷. The extent to which alterations in carbohydrate composition

affect the biodistribution and serum clearance of the antibody remains to be determined.

Agricultural-scale production

Clearly, if antibodies are to be used for therapeutic purposes, techniques for large-scale production have to be developed. The high capacity and flexibility of agricultural production offers several advantages for obtaining antibodies: genetically stable seed stocks of antibody-producing plants can be isolated and stored indefinitely at low cost and the seed stock can be converted into a harvest of any quantity of antibody within one growing season.

Although tobacco has been used as the principle research tool to initiate the study of antibodies in plants, there may be more appropriate plants for production. A variety of common crop plants can be used as the production host. Acreages of perennial forage crops could be generated by clonal propagation or from seed and harvested numerous times in a growing season. The choice of species may depend on the quantity and nature of contaminants encountered during purification. Some candidates are alfalfa, soybean, tomato and potato.

As large-scale production of antibodies is not yet commonplace, appropriate techniques for the purification of hundreds or thousands of grams have yet to be perfected. The cost of agriculturally-produced antibodies is likely to be considerably less than antibodies produced from hybridoma cells or ascites fluid. For example, if antibodies were expressed in soybean and constituted one per cent of total protein in soybean meal, a kilogram of antibody could, hypothetically, be produced for less than \$100 (US). This extrapolation is based on current costs for soybean production and does not take into account numerous hidden costs such as the cost of development and propagation of a sufficiently large and genetically characterized seed stock. In addition, the efficiency with which antibodies can be produced in specialized organs such as seeds or fruit is still not known.

Growth regulation

Plant growth and development is controlled by a limited number of low molecular weight hormones such as indoleacetic acid, ethylene, benzylaminopurine and a variety of more complex organic

molecules⁸. Little is known about the biosynthetic pathways or the mechanism of action of these hormones. However, by expression within the plant cell of monoclonal antibodies that recognise these hormones, it may be possible to evaluate developmental and metabolic events that are controlled by their free titre. Ideally, one would want to control the expression of the antibody as well as target expression to different organs or subcellular locations. In this way, activities of the hormone at various developmental stages could be unravelled.

Pathogen resistance

Antibodies against hormones are just one area where expression of an endogenous antibody could aid plant research. Another example is infection of plants by pathogens. Although many fungal, bacterial and viral pathogens have been characterized with respect to the genetics of host-pathogen interactions, very few have been thoroughly investigated at the biochemical level. In some instances, however, pathogen-related proteins or other organic molecules have been shown to be necessary for pathogenesis^{9,10}.

Expression of an intracellular antibody that binds antigens essential for pathogenesis may ameliorate the symptoms of the infection by reducing the functional titre. The advantage of this strategy is two-fold: first, it would not require isolation of genes involved in synthesis of the target antigen (as with anti-sense RNA expression); and second, pools of antigen which may be localized in subcellular compartments can be the specific target, leaving other pools unaffected. Clearly, the success of this approach will depend on a much more detailed understanding of the behaviour of antibodies in plants. Whereas antibodies have been successfully expressed intracellularly in both yeast and mammalian cells^{11,12}, attempts to assemble immunoglobulin chains in the cytosol of plants have been unsuccessful.

Current efforts are focusing on alternative methods which would by-pass the requirement for assembly of two immunoglobulin chains (for example, single chain antigen-binding constructs)¹³. In addition, attempts to localize an antigen-binding capacity to chloroplast and vacuole are in progress. Once we have a clear picture of the assembly, stability and functionality of targeted immunoglobulins, appropriate strategies for localized antigen binding can be devised.

Biofiltration

One of the key differences between plant cells and those of other organisms is the structure and characteristics of the surrounding cell wall. The mechanical strength and contiguous nature of plant cell walls is largely responsible for the rigidity of the entire plant. The diameter of pores in the

FASEB highlights

The Federation of American Societies for Experimental Biology (FASEB) annual meeting will be held in Washington, DC, next week. A micro-osmotic pump for slow-release drug delivery and a vertical tube gel apparatus will be among the many exhibits.

At FASEB, Beckman Instruments will be launching the programmable DU7500 diode array UV/visible spectrophotometer designed for microvolume and ultra-microvolume samples of up to 100 µl (*Reader Service No. 101*). The patented



DU7500: Beckman's next generation of UV/visible spectrophotometers.

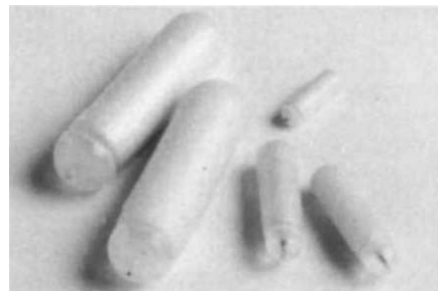
full spectrum quantitation applies all the data points in a scan to arrive at accurate and reliable component concentrations, says Beckman. Data are calculated using advanced vector quant maths. RediRead and RediScan modes allow the user to take readings or wavelength scans even when other measurements are in progress. A one-button prompt automatically sets up the new readings or scan, after which the interrupted research can be resumed. The DU7500 simplifies protein analysis by

cell wall imposes a restriction on the size of molecules that are freely permeable. This exclusion limit lies between 35 and 50 Å and corresponds to a molecular weight of less than 20,000 for a globular protein. Clearly, antibodies are too large to be freely permeable¹⁴. Consequently, expression of an antibody in a plant cell is equivalent to producing a binding and retention capacity within a semipermeable membrane. Any antigen with a molecular weight of less than 20,000 (for example, environmental pollutants, industrial by-products, pesticides and herbicides) might be collected and retained by a plant expressing an antibody that is functional *in situ*.

At present, research exploring the applications of biofilters is aimed at characterizing the functional properties of the antibody as it resides within the boundaries of the cell wall. Future efforts will be aimed at enhancing the functionality of antibodies in plants to enable catalytic

providing pre-selected parameters for Bradford (595 nm), Lowry (high sensitivity: 750 nm; low sensitivity: 500 nm), Biuret (540 nm) and direct UV method (280 nm). Kinetic analyses are run at single or multiple wavelengths: results can be displayed in five plot formats. Prices for the DU7500, which will be in action in booth 1312, range from \$15,000–25,000 (US), depending on configuration and choice of accessories.

To meet demands for an **implantable micro-osmotic pump** that can deliver a variety of bioactive compounds to animals weighing less than 10 grams, Alza Corporation has introduced the Alzet Model 1007D (*Reader Service No. 102*). Measuring just 17 mm in length and weighing 350 mg when empty, the Model 1007D provides the controlled administration of



Alza's micro-osmotic pumps provide sustained-release drug delivery.

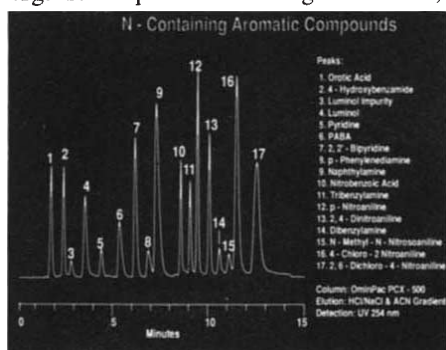
processing of molecules retained within the cell. □

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compounds such as peptides, growth factors and cytokines. The implantable infusion system has a 100- μ l capacity and pumps at a rate of 0.5 μ l h⁻¹ for a seven-day period. With key applications in the fields of neuroscience, immunology or cancer research, the Model 1007D allows the effects of agents with a short half life to be studied. Alza's full range of implantable pumps can be seen in booth 118.

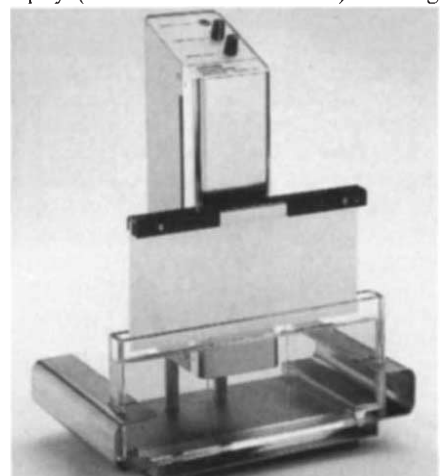
OmniPac PCX-500 HPLC columns, which combine reversed phase and ion exchange selectivities, allow the simultaneous separation of neutral and cationic organic compounds and inorganic cations,



OmniPac PCX-500 columns combine reversed phase and ion exchange selectivities.

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Camag of Switzerland has a handy Chromatogram Immersion Device III designed for efficient **postchromatographic derivatization** in thin-layer chromatography (*Reader Service No. 104*). Camag



Camag's immersion device — for reproducible postchromatographic derivatization.

says that by immersing the plate in the device, the reagent is uniformly transferred to the layer. The plate is immersed and withdrawn at an even speed to avoid

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Electrophoresis essentials

Polyanalyst is Buchler Instrument's **vertical tube gel apparatus**, which has an 18-tube capacity (*Reader Service No. 105*). The \$670 (US) device can be fitted with 3-mm ID tubes for running the first dimension of most two-dimensional gel protocols. For fractionation purposes, Polyanalyst can be fitted with universal grommets to accept gel tubes from 5-8-mm OD. An optional lower buffer chamber can be fitted to accommodate tubes up to 250 mm in length. This lower buffer reservoir is fitted with a water jacket to provide cooling when labile samples are being run, or when Polyanalyst is used for isoelectric focusing. For gel



Buchler Instrument's Polyanalyst vertical tube gel apparatus has an 18-tube capacity — see booth 1220.

polymerization, Polyanalyst is designed to allow tubes to be filled and gels to be directly polymerized in the unit. Polyanalyst, which will be on view in booth 1220, is supplied with levelling screws, 18 5-mm ID $\times$ 75-mm gel tubes and 18 destaining tubes for 5-mm ID gels.

Eliminate the need for vacuum pumps and cold traps with the **Easy Breeze air gel-drying system** from Hoefer Scientific (*Reader Service No. 106*). Easy Breeze is designed to dry six 20 $\times$ 20-cm or 24 8 $\times$ 10-cm gels simultaneously. The gel-drying system consists of an open-air oven and gel frames, which hold the gels tight and wrinkle-free, says Hoefer. After gels are loaded, warm air is blown across the

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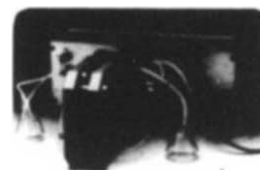
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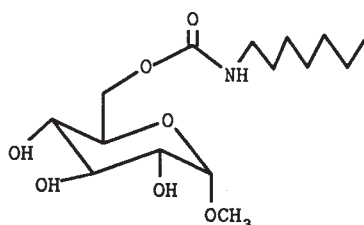
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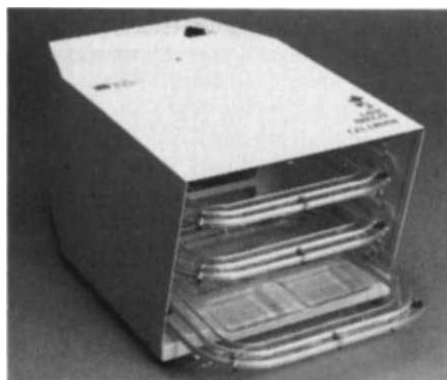
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The EC710 **volt-hour integrator** from E-C Apparatus Corporation, automates the end point of isoelectric focusing (IEF) or discontinuous buffer-style electrophoresis separations (*Reader Service No. 107*). The integrator connects to any power supply, IEF or electrophoresis system and provides a programmed empirical end-point determination based on volt hours applied



For end-point determinations of gel runs, E-C Apparatus offers a volt-hour integrator — the EC710.

during the run and is accurate to $\pm$ one per cent, says the company. The microprocessor-controlled EC710 can be programmed for up to one million volt hours and can be set to give digital readouts in either elapsed or remaining volt hours. An audible and visual alarm alerts the user to when there are zero hours remaining, after which the integrator will continue to register elapsed volt hours. See this useful and compact accessory, which measures 8 $\times$ 5.25 $\times$ 4.5 inches, in booth 1105.

NuSieve 3:1 is a new **agarose gel** from FMC BioProducts designed for the fine resolution of small DNA fragments, RNA and PCR products of less than 1 kb (*Reader Service No. 108*). NuSieve forms easy-to-handle gels with gel characteristics that include high gel strength and high melting temperature. NuSieve 3:1 agarose is tested for the absence of RNase and DNase activity and for the fine resolution of small DNA fragments. Visit FMC BioProducts in booth 1547.

RNA/DNA isolation/purification

Visit Collaborative Research in booth 523 and see its A-Plus kit for **mRNA isolation** (*Reader Service No. 109*). The A-Plus kit is supplied with all the reagents required for isolating and purifying mRNA directly from cell or tissue lysate. It includes a detailed protocol, buffers, columns, and oligo (dT)-cellulose for isolating high quality mRNA in four hours or less, says the company. The reagents are supplied in a ready-to-use form and are treated to eliminate ribonucleases to ensure high yields of mRNA.

Prep-A-Gene is a **DNA purification kit** from Bio-Rad designed for the rapid preparation of cloning-quality DNA (*Reader Service No. 110*). In just 20 minutes, says Bio-Rad, DNA can be purified from cleared lysates. As RNA and protein do not interfere with DNA binding to the Prep-A-Gene matrix, there is no need for RNase treatment, phenol extraction and other forms of sample preparation. Using this technique, DNA is eluted in a concentrated form with no precipitation, desalting or other post-treatment necessary. Bio-Rad point out that Prep-A-Gene has other applications: agarose gels with single-stranded and double-stranded DNA can be dissolved readily in the binding buffer; DNA free of RNA, organic agents, salts and proteins can be recovered in minutes; solutions of DNA can be desalted removing enzyme-inhibiting impurities; and unreactive, labelled nucleotide triphosphates, or small pieces of DNA such as linkers, can also be removed. Prep-A-Gene includes everything you need for DNA purification, although the matrix is available in 1- or 5-ml quantities. See Bio-Rad in booth 1111.

Cell science

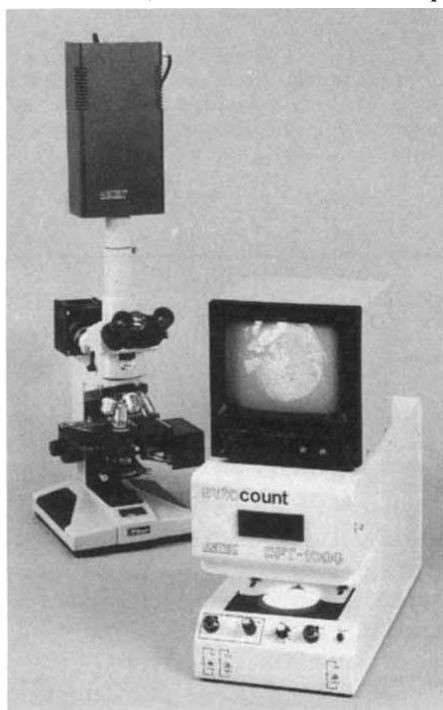
CytoFluor 2300 is the new **multiwell plate scanner** from Millipore for fluorescent assays, which has applications in cytotoxicity testing, *in vitro* toxicity testing and fluorescent ELISA assays (*Reader Service No. 111*). The fully automated computer-controlled CytoFluor 2300 accommodates 3-, 12-, 24- or 96-well plates. It is designed to quantify both the soluble and cell-



CytoFluor 2300: Millipore's multiplate scanner for fluorescent assays.

associated fluorescent signal, and can read a 96-well plate in less than 60 seconds, says Millipore. Multiple wavelength scanning is possible with CytoFluor. The instrument is supplied with five emission and four excitation filters: these allow the user to mix and match wavelengths to suit applications. The system scans from the bottom and so plate covers can be left in place to ensure sterility. For added flexibility, CytoFluor will also scan through the transparent Biopore membrane of Millicell-CM culture plate inserts. Data are reported as an Excel spreadsheet or as a comma separated values file. See the \$24,000 (US) CytoFluor system being put through its paces in booth 1039.

Researchers looking for ways to take the hard work out of counting objects under a microscope may be interested in Autocount, Dynatech's **automatic counter** which can be used to count bacterial colonies, plaques and cells (*Reader Service No. 112*). Autocount can count up



Automate counting operations with Autocount — new from Dynatech.

to 1,000 objects in a field, with totals displayed on a digital readout. By adjusting the aperture, the instrument can be used to isolate an object or group of objects for separate image counting. Size thresholds can be set, and scanned objects can be flagged with an illuminated dot. Autocount's electronic compensator automatically corrects counts by 15 per cent, says Dynatech, to account for any interference caused by stacking rings. Dynatech will be in booth 1036.

For the optimal growth of anchorage-dependent cells, Genex is offering bio-adhesive AdheraCell as a **solid-surface**

support (*Reader Service No. 113*). This natural adhesive protein securely attaches cells to many types of surfaces, providing, says Genex, optimal growth conditions for cell culture. AdheraCell is available in 2- and 5-mg quantities for \$95 and \$230 (US), respectively. Genex will be exhibiting its new products to aid biomedical research in booth 244.

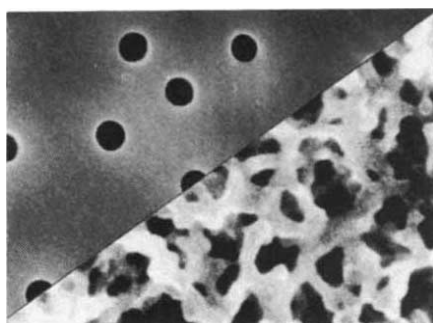
Antibody products

Bioproducts for Science has made available BioBind-G **agarose for affinity chromatography** applications (*Reader Service No. 114*). BioBind-G agarose, a recombinant type-2 form of streptococcal Protein G, is covalently immobilized on Sepharose by the cyanogen bromide method. The company sells BioBind-G agarose in 5-ml quantities as a 50 per cent gel slurry in phosphate-buffered saline at pH7 and with thimerosal as a preservative. For convenience, BPS also offers BioBind-G ReadiPak columns. Each 1-ml column contains 2.5 mg of agarose. Stated applications include the purification of IgG antibodies from ascites fluid, hybridoma cell culture supernatant, or serum containing polyclonal antibodies. In addition, BioBind-G can be used for immunoprecipitation procedures using monoclonal and polyclonal antibodies, isolation of immune complexes, and the removal of IgG antibodies from serum samples. See BPS at FASEB in booth 950.

Zymed Laboratories, exhibiting in booth 829, has added to its existing line of **ready-to-use primary antibodies** for immunohistochemical and immunocytochemical procedures (*Reader Service No. 115*). The expanded line now includes ACTH, B cell, oestradiol, gastrin, glucagon, insulin, lactoferrin, myosin, papillomavirus, progesterone, serotonin, somatostatin, testosterone, transferrin and vasoactive intestinal polypeptide. The antibodies are supplied in 6-ml quantities, which is enough for 60 paraffin-embedded slides, says Zymed. Prices range from \$30 to \$85 (US).

Filtering aids

According to Poretics Corporation, exhibiting in booth 1553, its improved



Polycarbonate membrane screen filters (top), tortuous pore membrane filters (bottom).

polycarbonate membrane screen filters offer low adsorption and absorption for accurate separations and fractionations (*Reader Service No. 116*). Electron microscopy reveals that the membranes are two-dimensional microporous screens with cylindrical, straight-through pores and a flat, smooth non-hygroscopic surface for sample capture. Polycarbonate filters are available in pore sizes ranging from 0.01 μm to 14 μm and in standard circle sizes that range from 13 mm to 293 mm in diameter.

Whatman Scientific has designed a **disposable microfiltration device** called the Puradisc 25PP, which can be used with both aqueous solutions and organic solvents (*Reader Service No. 117*). Puradisc 25PP can be used to filter small volumes (up to 100 ml) with low hold-up volumes, says Whatman. The filtration device

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Whatman's microfiltration device for aqueous and solvent applications.

comes with a compact polypropylene housing, a 25-mm filter, and a standard luerlock fitting for attachment to a syringe. Filters are available with porosities of either 0.2 or 0.45 μm . The unit can be autoclaved. The filtration device is supplied in quantities of 50 or 200. See Whatman in booth 1434.

Lab instrumentation

Step by Isco's booth, number 1133, and see the Foxy 200 **programmable fraction collector** for preparative HPLC and

process LC (*Reader Service No. 118*). Using the Foxy 200, fractions can be sized by time, drop counting or volumetric signal from a pump. The instrument collects peaks selectively by monitoring the slope and/or level of a detector signal, as well as by the expected retention time. Fractions can be collected in small-volume tubes with non-peak material diverted to larger containers or a waste station. The instrument accommodates up to 288 tubes or 26 outboard containers. Easy-to-load racks allow a choice of nine standard tube and vial sizes ranging from microcentrifuge tubes to 28-mm scintillation vials.



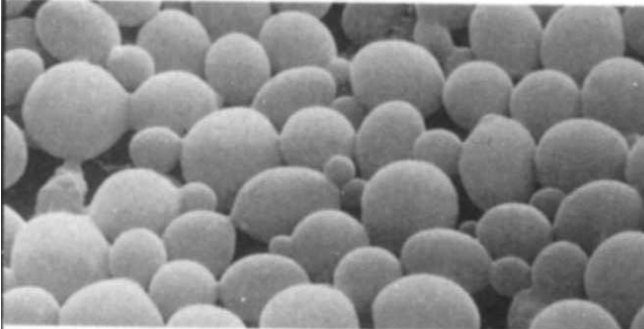
Isco's Foxy 200 fraction collector for multi-mode peak collection with HPLC and LC applications.

Foxy 200 offers menu-based input of collection parameters and the storage of run protocols. An optional accessory controller allows the Foxy 200 to interact with pumps and valves, providing complete automation of LC.

Du Pont has introduced two new Sorvall space-saving **micro-ultracentrifuges** (*Reader Service No. 119*). The RC-M120 micro-ultracentrifuge has a maximum speed of 120,000 r.p.m. (600,000g), while the RC-M100 has a top speed of 100,000 r.p.m. (541,000g) and can reach maximum speed in just 1.5 minutes, says the company. For some protocols, Du Pont says, run times can be reduced by up to 80 per cent. Lipoproteins, which typically take up to 20 hours to separate using conventional ultracentrifuges, now take 2–3 hours. Both models feature high-efficiency drive systems and a direct, brushless motor design, eliminating the need for external cooling devices. The Sorvall micro-ultracentrifuges have a 40-ml sample capacity. See Du Pont at FASEB in booth 1124. □

These notes are compiled by Diane Gershon from information provided by the manufacturers. To obtain further details, use the reader service card bound inside the journal. Prices quoted are sometimes nominal, and apply only within the country indicated.





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